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# Sleep And Activity Problems In Mouse Models Of Neurodevelopmental Disorders

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# Sleep And Activity Problems In Mouse Models Of Neurodevelopmental Disorders

## **Abstract**

Adequate sleep is important for long-term health and day-to-day function. Compared to the general population, patients diagnosed with neurodevelopmental disorders have substantially higher prevalence of sleep, activity, and circadian problems, dramatically affecting their quality of life, and potentially exacerbating other adverse symptomologies. Despite this, the neurobiological underpinnings of sleep problems in neurodevelopmental disorders remain unknown, and accurate rodent models capable of recapitulating human sleep and activity problems are lacking. In this dissertation, I investigate sleep, activity, and circadian rhythms in genetic mouse models of human neurodevelopmental disorders, with a focus on autism spectrum disorder (ASD). In Chapter 1, I review the importance of—and mechanisms contributing to—sleep/wake regulation, sleep problems in neurodevelopmental disorders, and the utility of rodent genetic models to address these problems. In Chapter 2, I investigate hyperactivity and male-specific sleep deficits found in the 16p11.2 del/+ chromosomal copy number variation mouse model of neurodevelopmental disorders (Angelakos et al., 2016). In Chapter 3, I highlight REM sleep reductions and altered electroencephalography (EEG) spectra in the SYGNAP1+/- mouse model of intellectual disability and ASD. In Chapter 4, I discuss home-cage hypoactivity observed in four different mouse models of ASD.

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# SLEEP AND ACTIVITY PROBLEMS IN MOUSE MODELS OF NEURODEVELOPMENTAL DISORDERS

Christopher Caleb Angelakos

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in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

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Degree of Doctor of Philosophy

2017

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# ABSTRACT

## SLEEP AND ACTIVITY PROBLEMS IN MOUSE MODELS OF NEURODEVELOPMENTAL DISORDERS

Christopher Caleb Angelakos

Ted Abel

Adequate sleep is important for long-term health and day-to-day function. Compared to the general population, patients diagnosed with neurodevelopmental disorders have substantially higher prevalence of sleep, activity, and circadian problems, dramatically affecting their quality of life, and potentially exacerbating other adverse symptomologies. Despite this, the neurobiological underpinnings of sleep problems in neurodevelopmental disorders remain unknown, and accurate rodent models capable of recapitulating human sleep and activity problems are lacking. In this dissertation, I investigate sleep, activity, and circadian rhythms in genetic mouse models of human neurodevelopmental disorders, with a focus on autism spectrum disorder (ASD). In **Chapter 1**, I review the importance of—and mechanisms contributing to—sleep/wake regulation, sleep problems in neurodevelopmental disorders, and the utility of rodent genetic models to address these problems. In **Chapter 2**, I investigate hyperactivity and male-specific sleep deficits found in the 16p11.2 del/+ chromosomal copy number variation mouse model of neurodevelopmental disorders (Angelakos et al., 2016). In **Chapter 3**, I highlight REM sleep reductions and altered electroencephalography (EEG) spectra in the SYGNAP1<sup>+/-</sup> mouse model of intellectual disability and ASD. In **Chapter 4**, I discuss home-cage hypoactivity observed in four different mouse models of ASD.

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## **CHAPTER 1: Mechanisms and functions of sleep and circadian rhythms**

### **Abstract**

Sleep is highly conserved across the animal kingdom. A diverse range of organisms including nematodes, flies, fish, reptiles, birds, and mammals exhibit sleep or sleep-like behavioral states. The fact that sleep is so evolutionarily conserved across phylogeny, and humans spend nearly one-third of their lives in this vulnerable state, supports its importance. Indeed, adequate sleep has been shown to be important for health, metabolism, learning, cognition, mood, synaptic scaling, immune system function, hormone release, motor performance, and more. However, in autism spectrum disorder and related neurodevelopmental disorders, sleep is disturbed. In this chapter, I review some important functions of sleep and mechanisms underlying normal sleep and circadian regulation. I also summarize sleep issues highly prevalent in neurodevelopmental disorders, with a focus on autism spectrum disorder (ASD). Finally, I address the utility and need for genetic mouse models of neurodevelopmental disorders capable of reproducing sleep, activity, and circadian problems found in human neurodevelopmental disorders.

## **1.1 Mechanisms of sleep and circadian regulation**

Sleep is a perplexing and highly conserved behavioral phenomenon. It is a reversible period of altered consciousness marked by behavioral quiescence, heightened arousal threshold, and in humans and other animals with a sufficiently developed cortex—altered brain firing patterns that can be measured by electroencephalography (Haas, 2003). Through polysomnography—the combination of electroencephalography (EEG) (brain waves), electromyography (EMG) (skeletal muscle activity), and sometimes electrooculography (EOG) (eye movements)—three broad sleep/wake stages can be ascertained: wake, non-rapid eye movement sleep (NREM), and rapid eye movement sleep (REM). Wake is characterized by high frequency, low amplitude waveforms in the EEG with frequent muscle activity in the EMG. NREM sleep is characterized by low frequency, high amplitude (synchronized) waveforms in the EEG and minimal muscle tone in the EMG. For these reasons, NREM sleep is also referred to as slow wave sleep. REM sleep is characterized by high frequency, low amplitude waveforms in the EEG, similar in its desynchronized activity to wake, but with an increase of power in the theta frequency (4-8Hz). Distinct from wake, however, REM sleep is also characterized by rapid eye movements and a complete loss of muscle tone in the EMG (Dement & Kleitman, 1957). Sleep and wakefulness is believed to be regulated by two distinct overarching modulators: circadian rhythms (also known as “Process C”) and homeostatic drive (also known as “process S”) (Borbély, 1982). These processes work in concert to wake us in the morning and drive us toward sleep in the evening.

### **1.1.1 Circadian rhythms and regulation**

Circadian rhythmicity is an ~24-hour endogenous biological process (Czeisler et

al., 1999) that is entrained by environmental cues to the 24-hour rhythm of the rotating earth (J C Dunlap, 1999). The endogenous biological clock is robust, tightly regulated, and persists independent of the prior amount of time spent awake/asleep. Genomics studies indicate that anywhere from 2% (Duffield et al., 2002) to 8-10% (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002) of the genome is circadian regulated, and approximately 40% of coding genes oscillate in at least one organ (Zhang et al., 2014). In mammals, the “master” circadian clock is located within the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972; Welsh et al., 1995). The SCN receives direct environmental light cues via the retinohypothalamic tract, and in turn signals circadian timing peripherally throughout the body via neural outputs, downstream endocrine secretion, and by SCN-driven circadian regulation of body temperature (Okamura, 2007; Dibner et al., 2010; Buhr & Takahashi, 2013). The molecular clock is driven by a transcriptional-translational feedback loop of core clock molecules. The basic helix-loop-helix-PAS-containing transcription factors CLOCK and BMAL1 heterodimerize and bind to Enhancer box sequences in the promoters of the negative regulators mPER and mCRY, driving their transcription. mPER and mCRY in turn feedback onto CLOCK:BMAL1, inhibiting their transcription (Reppert & Weaver, 2002). In addition to this molecular negative feedback loop, a separate feedback loop involving RAR-related orphan receptor alpha (ROR $\alpha$ ) and Rev-Erba maintains the circadian regulation of BMAL1 by activating and repressing BMAL1 transcription, respectively (Preitner, Damiola, Luis-Lopez-Molina, et al., 2002). Numerous other transcriptional activators and repressors have been identified that play supplementary but important roles in maintaining precise circadian timing (for recent reviews, see: Golombek & Rosenstein, 2010; Buhr & Takahashi, 2013; Partch et al., 2014).



### **1.1.2 Sleep homeostat (Process S)**

A second process known as the sleep homeostat (or Process S), is dependent upon prior time spent awake or asleep (A A Borbély, 1982). The longer we stay awake, the more intense is the drive to sleep. This is evident after sleep disruption, chronic sleep restriction, or total sleep deprivation following an all-nighter (Klerman & Dijk, 2005; A. C. Reynolds & Banks, 2010). Conversely, the need to sleep decreases as the prior duration of sleep increases. Behavioral and physiological correlates following sleep deprivation provide evidence for the existence of a sleep homeostat. Following sleep deprivation, there is an increase of sleep time and slow wave activity (0.5-4.0Hz) relative to the non-sleep restricted baseline day. Even without sleep deprivation, slow wave activity is modulated as a function of prior wakefulness. Slow wave activity increases as a function of time spent awake, and declines progressively over subsequent NREM bouts (Borbély et al., 1981; Dijk et al., 1991; Achermann et al., 1993; Dijk et al., 1993; Campbell et al., 2006). In addition to physiological correlates, there are also molecular homeostatic correlates. Interstitial concentration of the molecule adenosine increases with wakefulness, and decreases with sleep. Following sleep deprivation, adenosine tone increases in the basal forebrain and cortex relative to baseline levels, and declines during recovery sleep (Porkka-Heiskanen et al., 1997; Porkka-Heiskanen et al., 2000). In rats, stimulation of the G<sub>i</sub>-coupled adenosine A1 receptor increases NREM slow wave activity, mimicking the effects observed following sleep deprivation (Ticho & Radulovacki, 1991; Benington et al., 1995), while A1 receptor antagonism reduces recovery sleep and slow wave activity (Gass et al., 2009). Caffeine, the world's most widely consumed psychoactive drug due to its wake-promoting effects, is a potent adenosine receptor

antagonist (Smits et al., 1987; Fredholm, 1995). Numerous other molecules, including glucocorticoids and peptide hormones likely influence the sleep homeostat; however, at present time, adenosinergic contribution is the most extensively studied.

### **1.1.3 Sleep-state switching**

In addition to circadian rhythms and homeostatic drive, which regulate sleep and wakefulness broadly, myriad interconnected circuits and transmitter systems work in interplay to regulate the transitions between wake, NREM sleep, and REM sleep (reviewed in Fort et al., 2009; Saper et al., 2010). Briefly, and certainly not exclusively, arousal networks include nuclei of the reticular formation of the brainstem (Moruzzi & Magoun, 1949), cholinergic neurons of the pedunculopontine tegmental nuclei (PPT), laterodorsal tegmental nuclei (LDT), and basal forebrain (el Mansari et al., 1989; Cape & Jones, 2000; Lee et al., 2005a; Kaur et al., 2008; Boucetta & Jones, 2009; Hassani et al., 2009a), noradrenergic projections from the locus coeruleus (Aston-Jones & Bloom, 1981), serotonergic efferents from the dorsal and median raphe (Monti, 2011), dopaminergic neurons in the ventral periaqueductal gray (Lu et al., 2006a), dorsal raphe (Cho et al., 2017) and ventral tegmental area (Eban-Rothschild et al., 2016), histaminergic neurons of the tuberomammillary nucleus (Steininger et al., 1999; Takahashi et al., 2006), and orexinergic neurons of the lateral hypothalamus (Lin et al., 1999; Lee et al., 2005b; Adamantidis et al., 2007; Carter et al., 2009). NREM sleep-promoting networks include a subset of GABAergic neurons of the parafacial zone of the brainstem (Anaclet et al., 2012, 2014) and the ventrolateral preoptic nucleus (Nauta, 1946; McGinty & Serman, 1968; Sherin et al., 1996; Lu et al., 2000; Chung et al., 2017) and median preoptic nucleus of the hypothalamus (Suntsova et al., 2002; Gvilia et al., 2006; Chung et al., 2017), which

project to and receive extensive inputs from many of the wake-promoting regions mentioned above (Chou et al., 2002; Uschakov et al., 2007). Finally, REM promoting circuitry includes subset of GABAergic neurons in the ventral medulla (Weber et al., 2015), as well as the reciprocally interacting REM-ON sublaterodorsal nucleus (SLD) and REM-OFF GABAergic neurons of the ventrolateral periaqueductal gray and lateral pontine tegmentum (vIPAG-LPT) (Sastre et al., 1996; Boissard et al., 2002; Lu et al., 2006b; Luppi et al., 2006). Additional REM sleep-modulating networks exist in the cholinergic neurons of the PPT and LDT (Webster & Jones, 1988; el Mansari et al., 1989), as well as melanin-concentrating hormone-expressing neurons of the lateral and posterior hypothalamus (Verret et al., 2003; Hassani, Lee, & Jones, 2009; Jengo et al., 2013). Clearly, the regulation of sleep and wake is an intricate and complicated process resulting from the delicate interaction of dozens of brain regions and transmitter systems. Aberrant neural signaling in a number of brain regions can lead to misregulation of sleep and circadian rhythms. Consequences of altered sleep and circadian rhythms are discussed in the next section.

## **1.2 Functions of sleep and circadian rhythms, and consequences of their dysfunction**

The function of sleep is still debated. In truth, functions of sleep are likely multifaceted, situation-dependent, and different across development. Sleep is less energetically demanding than wake, bringing about decreased body temperature, heart rate, and oxygen consumption (Kleitman & Ramsaroop, 1948). Thus, sleep has been proposed to be restorative, allowing for the clearance of detrimental oxygen free radicals and  $\beta$ -amyloid that may accumulate during the metabolically taxing state of an active, awake brain (Reimund, 1994; Xie et al., 2013). Sleep is intimately connected with immune

system function (Besedovsky et al., 2012), is affected and modulated by cytokines (Opp, 2005), and is believed to play an important role in recovery from illness (Toth et al., 1993; Imeri & Opp, 2009). Sleep also plays a role in growth and development. Slow wave sleep promotes release of growth hormone, which curtails with age (Takahashi et al., 1968; Van Cauter et al., 2000). Adequate sleep in infants is related to cognitive performance and language-learning (Gómez et al., 2006; Touchette et al., 2007; Beebe, 2011). Moreover, sleep is crucial for the consolidation of both procedural and declarative memories (Diekelmann & Born, 2010; Abel et al., 2013). During the subsequent sleep episode following explorative learning, hippocampal place cells increase their firing rate and “replay” the pattern of place cell activity experienced during exploration, suggesting a role for sleep in spatial consolidation (Pavlides & Winson, 1989; Skaggs & McNaughton, 1996). At the synaptic level, sleep is believed to modulate spine growth and connectivity, allowing for the remembering of useful information and the forgetting of non-essential material (Tononi & Cirelli, 2006; G. Yang et al., 2014). Still, the most striking evidence for the function and importance of sleep and circadian rhythms comes from the severe consequences resulting from their disruption.

### **1.2.1 Consequences of inadequate sleep**

Insufficient sleep, a common problem in modern society, is concomitant with numerous health and cognitive issues. Chronic sleep loss is associated with an increased prevalence for obesity, diabetes, hypertension, and heart attacks (Taheri et al., 2004; Spiegel et al., 2005; Mullington et al., 2009), as well as shorter lifespans (Kripke et al., 2002; Patel et al., 2004; Tamakoshi et al., 2004). Insufficient sleep increases plasma concentrations of cortisol and pro-inflammatory cytokines, alters glucose metabolism and

impacts insulin sensitivity, and modifies levels of leptin and ghrelin (Vgontzas et al., 1997; Spiegel et al., 1999; Knutson et al., 2007). Psychologically, long-term sleep deficit is positively correlated with depression, anxiety, and alcohol abuse (Pilcher & Huffcutt, 1996). Total sleep deprivation, partial sleep deprivation, and chronic sleep restriction all impair performance on a number of neurocognitive tasks of executive function and working memory. Moreover, performance generally worsens proportional to the amount of sleep deprivation and the length of the cognitive task (Van Dongen et al., 2003; Durmer & Dinges, 2005). Following learning, restricted sleep and total sleep deprivation impairs memory consolidation and subsequent recall (Walker, 2008; Diekelmann & Born, 2010; Abel et al., 2013). Clearly, the effects of insufficient sleep in humans are substantial and widespread.

Work from our lab, in mice, has uncovered cellular and molecular consequences of acute sleep deprivation, especially as it relates to the consolidation of hippocampus-dependent memories. Six hours of sleep deprivation following learning impairs spatial object recognition and contextual fear memory 24 hours later (Graves et al., 2003; Halassa et al., 2009). Sleep deprivation decreases levels of cAMP—a second messenger important for protein synthesis and long-term memory formation—by increasing levels of phosphodiesterase isoform 4A5 (PDE4A5), an enzyme that breaks down cAMP (Vecsey et al., 2009). During sleep deprivation, pharmacogenetic augmentation of cAMP or blockade of hippocampal PDE4A5 function prevents spatial memory impairments (Vecsey et al., 2009; Havekes et al., 2014). Moreover, acute sleep deprivation attenuates translational mechanisms by interfering with the mammalian target of rapamycin (mTOR) pathway, preventing normal memory consolidation (Tudor et al., 2016). At the cellular level, acute sleep deprivation elevates cofilin activity and decreases dendritic spine length

and density in hippocampus region CA1, which is normalized by recovery sleep (Havekes et al., 2016). Further, 6 hours of sleep deprivation impairs certain forms of long-term potentiation in the hippocampus, the presumptive cellular correlate for learning (Vecsey et al., 2009). Thus, even one night of acute sleep deprivation has molecular consequences and is capable of altering neural structure and connectivity.

### **1.2.2 Consequences of circadian dysfunction**

Proper alignment between endogenous circadian clocks and exogenous environmental stimuli are important for health and function. It is estimated that 15% of all metabolites are under circadian control (Dallmann et al., 2012), and metabolic diseases are associated with circadian disturbances (reviewed in: Delezie & Challet, 2011). Circadian disruption caused by shift work is associated with obesity, heightened levels of triglycerides, type 2 diabetes, and an increased risk for cancer and cardiovascular disease (Bøggild & Knutsson, 1999; Davis et al., 2001; Karlsson et al., 2001; Schernhammer et al., 2001, 2003; Pan, et al., 2011). In addition to long-term health detriments, shift work is also associated with fatigue resulting in significantly more work accidents (Smith et al., 1994) and increased work errors due to sleepiness (Åkerstedt, 1988).

Important circadian influences on metabolism and health have also been demonstrated in cyanobacteria, *Drosophila*, and rodents. Strains of cyanobacteria whose internal oscillator closely match the external environment out-compete strains of cyanobacteria that do not oscillate in tune with the environment (Woelfle et al., 2004). *Drosophila* with impaired circadian clock function have decreased reproductive success (Beaver et al., 2002), altered carbohydrate and lipid homeostasis (Seay & Thummel, 2011), and are more sensitive to infection (Shirasu-Hiza et al., 2007). In mice, CLOCK

gene mutants are hyperphagic, have altered glucose metabolism, and become obese (Turek et al., 2005). Chronically “jet lagged” wildtype mice who undergo an 8 hour phase advance at the beginning of each week develop leptin resistance and become obese (Kettner et al., 2015). Moreover, mice receiving a chronic jet lag protocol of four consecutive weekly 6-hour phase advances have impaired immune system function, hypothermia, and a 4-fold increased mortality rate following LPS injection (Castanon-Cervantes et al., 2010). Additionally, tau-shortened hamster mutants kept on a normal 12h:12h light:dark cycle develop cardiomyopathies, renal disease, and die prematurely. Importantly, if the light:dark cycle is adjusted to match the endogenous circadian rhythm of these hamster mutants with short endogenous rhythms, no detriments were observed in these organs (Martino et al., 2008).

In humans, there are five currently recognized circadian disorders: delayed sleep phase disorder, advanced sleep phase disorder, irregular sleep-wake disorder, non-24-hour sleep-wake disorder, and shift work sleep disorder. Circadian disorders are comorbid with ADHD and depression (Bunney, 2000; Baird et al., 2012). Much of the problems resulting from circadian disorders are societal in nature, as afflicted individuals may have trouble going to school or holding jobs requiring a standard 9-5 schedule without constantly suffering from sleepiness, fatigue, headaches, and cognitive impairments (Okawa and Uchiyama, 2007).

### **1.3 Summary of the functional importance of sleep and circadian rhythms**

In summary, adequate sleep and precisely timed circadian rhythmicity is important for health, metabolism, immune function, learning, cognition, mood, synaptic scaling,

hormone release, motor performance, and more. Sleep and circadian problems result in profound behavioral, cellular, and molecular consequences, affecting quality of life and long-term health. Much of what we know about the circuitry and molecular mechanisms underlying normal and deficient sleep and circadian rhythmicity comes from studies in rodent models. These factors motivated my dissertation work—to investigate sleep, activity, and circadian rhythms in mouse genetic models of neurodevelopmental disorders—a patient population with profoundly enhanced sleep issues relative to the general population. The long-term goal of these studies is to identify accurate genetic models capable of recapitulating some of the diverse sleep and activity issues that cause substantial burden to the majority of individuals diagnosed with a neurodevelopmental disorder.

#### **1.4 Sleep and circadian problems in autism spectrum disorders and related neurodevelopmental disorders**

Autism spectrum disorder (ASD) is a multifaceted disorder of neural development, affecting an estimated 1 in 68 children born today. ASD is strongly sex-biased, with males 4.5 times more likely than females to receive an ASD diagnosis (Christensen et al., 2016), even with the same genetic insult (Robinson et al., 2013; Jacquemont et al., 2014). Patients diagnosed with ASD typically exhibit social deficits, communication problems, and repetitive behaviors. In addition to these “core” ASD symptomologies, it is now recognized that sleep problems are a hallmark of ASD, as well as other neurodevelopmental disorders (for reviews, see: Richdale & Schreck, 2009; Glickman, 2010; Robinson-Shelton & Malow, 2016; Souders et al., 2017).



The prevalence of sleep problems in neurodevelopmental disorders are estimated to be as high as 80%, representing a 2-3 fold increase over the typically developing population (Robinson-Shelton & Malow, 2016; Souders et al., 2017). The most common sleep problem in ASD is insomnia, which is 10x more likely in children with ASD than in children without a neurodevelopmental disorder diagnosis (Sivertsen et al., 2012). Other sleep problems prevalent in ASDs include difficulties falling asleep, difficulties staying asleep (increased night awakenings), and early morning rising (Limoges et al., 2005; Malow et al., 2006; Miano et al., 2007; Goldman et al., 2009; Richdale & Schreck, 2009; Souders et al., 2009; Picchioni et al., 2014). ASD patients have been reported to have decreased REM sleep time and reduced rapid-eye movements during REM sleep (Limoges et al., 2005; Buckley et al., 2010). On top of the direct consequences of altered sleep in ASD, sleep problems are correlated with the severity of other negative ASD symptomologies, and have been estimated to account for 22-32% of the variance in behavioral problems in ASD (Park et al., 2012; Mazurek & Sohl, 2016).

Insomnia and early morning wakings in ASD suggest circadian abnormalities. Melatonin is a circadian-regulated hormone produced by the pineal gland that regulates timing of sleep and wakefulness. Indeed, numerous studies have reported altered melatonin synthesis, concentration, and circadian profile in ASD patients relative to typically developing controls (Ritvo et al., 1993; Nir et al., 1995; Kulman et al., 2000; Tordjman et al., 2005; Bourgeron, 2007; Melke et al., 2008; Mulder et al., 2010). Nocturnal melatonin has helped mitigate insomnia in some ASD patients (Rossignol & Frye, 2011; Cortesi et al., 2012; Cuomo et al., 2017), but may result in earlier morning awakenings (Gringras et al., 2012). Further, meta-analyses of randomized, double-blinded, and placebo-controlled studies of nocturnal melatonin therapy revealed no significant

improvements in night-time awakenings in ASD patients (Rossignol & Frye, 2011; Cuomo et al., 2017).

## 1.5 Mouse genetic models for studying sleep and circadian rhythms

The benefits of using genetically-modified mice to study sleep and circadian rhythms are multiple. Mice have brains with similar structure, circuitry, and regional functionality as humans. Unlike simpler model organisms such as *C. elegans* and *Drosophila*, polysomnography can be performed in rodents, they exhibit REM sleep, and they demonstrate rebound slow wave activity and sleep time following sleep deprivation (Franken et al., 1991). Moreover, experimental protocols for studying sleep and circadian rhythms in mice are well-defined. Most importantly, among animals amenable to powerful genetic manipulations, mice are the closest phylogenetic relative to humans.

Despite the fact that dozens of genetic mouse models of ASD have been generated and the high prevalence of sleep problems in human patients, at the present time, only two studies have quantified sleep and circadian rhythms in any rodent genetic model of ASD (Thomas et al., 2016, 2017). In the following chapters, I will discuss my dissertation research outlining sleep, activity, and circadian alterations in multiple rodent genetic models of neurodevelopmental disorders. In **Chapter 2**, I describe male-specific sleep deficits in the 16p11.2 del/+ mouse model of neurodevelopmental disorders. In **Chapter 3**, I show altered REM sleep and cortical EEG spectra in the *Syngap1*<sup>+/-</sup> mouse model of intellectual disability and ASD. In **Chapter 4**, I present an unexpected, but shared home-cage hypoactivity phenotype across four different mouse models of ASD. Utilizing the models outlined in this dissertation, we eventually hope to 1) identify neural circuits

and molecular etiologies of the sleep, activity, and circadian alterations described herein, and 2) test potential treatment strategies—with the ultimate goal of improving quality of life for both patients diagnosed with neurodevelopmental disorders and their caretakers.

**Author Contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Ted Abel.

## **CHAPTER 2: Hyperactivity and male-specific sleep deficits in the 16p11.2 deletion mouse model of autism**

### **Abstract**

Sleep disturbances and hyperactivity are prevalent in several neurodevelopmental disorders, including autism spectrum disorders (ASDs) and attention deficit-hyperactivity disorder (ADHD). Evidence from genome-wide association studies indicates that chromosomal copy number variations (CNVs) are associated with increased prevalence of these neurodevelopmental disorders. In particular, CNVs in chromosomal region 16p11.2 profoundly increase the risk for ASD and ADHD, disorders that are more common in males than females. We hypothesized that mice hemizygous for the 16p11.2 deletion (16p11.2 del/+) would exhibit sex-specific sleep and activity alterations. To test this hypothesis, we recorded activity patterns using infrared beam breaks in the home-cage of adult male and female 16p11.2 del/+ and wildtype (WT) littermates. In comparison to controls, we found that both male and female 16p11.2 del/+ mice exhibited robust home-cage hyperactivity. In additional experiments, sleep was assessed by polysomnography over a 24-hour period. 16p11.2 del/+ male, but not female mice, exhibited significantly more time awake and significantly less time in non-rapid-eye-movement (NREM) sleep during the 24-hour period than wildtype littermates. Analysis of bouts of sleep and wakefulness revealed that 16p11.2 del/+ males, but not females, spent a significantly greater proportion of wake time in long bouts of consolidated wakefulness (greater than 42 minutes in duration) compared to genetic controls. These changes in hyperactivity, wake time, and wake time distribution in the males resemble sleep disturbances observed in human ASD and ADHD patients, suggesting that the 16p11.2 del/+ mouse model may

be a useful genetic model for studying sleep and activity problems in human neurodevelopmental disorders.

## Introduction

Sleep and activity problems are extremely prevalent in autism spectrum disorders (ASDs) and attention-deficit/hyperactivity disorder (ADHD), yet they remain poorly understood. Disrupted sleep affects up to 80% of ASD patients and 55% of children with ADHD, compared to only 7-30% in the control population (Ivanenko & Johnson, 2008; Goldman et al., 2011; Cohen et al., 2014; Kirov & Brand, 2014). The most commonly reported sleep issues in these neurodevelopmental disorders include insomnia, delayed sleep onset, and increased night awakening (Ivanenko & Johnson, 2008; Ming & Walters, 2009; A. M. Reynolds & Malow, 2011). These subjective observations have been supported by objective polysomnography and actigraphy sleep recordings (Miano et al., 2007; Cortese et al., 2009; Goldman et al., 2009; Souders et al., 2009). In addition, sleep problems are positively correlated with the severity of core ASD symptomology including communication deficits, withdrawal, and repetitive and stereotyped behavior (Cortesi et al., 2010; Park et al., 2012). Despite this, few well-controlled studies have addressed the neurobiological underpinnings of sleep problems in these disorders. The high prevalence of sleep problems in ASDs and ADHD and the impact of disrupted sleep on symptomology highlight the utility of identifying an appropriate genetic model with which to test potential treatments and elucidate mechanisms underlying these disorders.

Increasing evidence from genome-wide association studies suggests that chromosomal copy number variations (CNVs) are significantly enhanced in many neurodevelopmental disorders (Sebat et al., 2007; Grayton et al., 2012). In particular, hemideletion in chromosomal region 16p11.2 profoundly increases the risk for several neurodevelopmental disorders, including ASD and ADHD, even when controlling for the

high comorbidity between these disorders (Hanson et al., 2014). 16p11.2 hemideletion is associated with an estimated 0.6% of all ASD diagnoses (Weiss et al., 2008), and 16p11.2 hemideletion patients display cognitive deficits and other ASD symptomology even if they do not meet the criteria for ASD diagnosis (Stefansson et al., 2014). Unlike many factors believed to contribute to neurodevelopmental disorders, the 16p11.2 chromosomal region is highly conserved in the syntenic 7qF3 region in the mouse, and thus copy number variation in this region can be accurately modeled. To date, three different mouse lines of 16p11.2 hemideletion have been created, varying in the size of the deletion as well as genetic background of the mice (Horev et al., 2011; Portmann et al., 2014; Arbogast et al., 2016). Indeed, 16p11.2 hemideletion (16p11.2 del/+) mice have deficits in brain structure, cognition, and communication (Horev et al., 2011; Portmann et al., 2014; Pucilowska et al., 2015; Brunner et al., 2015; Yang et al., 2015a; Yang et al., 2015b; Arbogast et al., 2016).

ASDs and ADHD also show significant sex bias risk, but the mechanisms contributing to this remain unknown. Males are 4 times more likely than females to be diagnosed with ASD (Werling & Geschwind, 2013) and 3 times more likely to be diagnosed with ADHD (Schneider & Eisenberg, 2006). For this reason, developing genetic models that also demonstrate this risk bias is important for construct validity. Previous studies utilizing the 16p11.2 del/+ mouse model have not directly compared males and females (Brunner et al., 2015; Pucilowska et al., 2015; Yang et al., 2015a; Yang et al., 2015b; Arbogast et al., 2016). In this study, we assessed home-cage activity using infrared beam breaks and sleep/wake behavior using polysomnography in both male and female adult 16p11.2 del/+ mice. We found robust home-cage hyperactivity across the diurnal cycle in



both male and female 16p11.2 del/+ mice compared to sex-matched wildtype littermates. In addition, we found male-specific sleep/wake decrements in total sleep time and distribution of wakefulness. In 16p11.2 del/+ males, but not females, the proportion of wake time distributed in long bouts of continuous wakefulness was significantly greater than in sex-matched WT littermates. When compared to sex-matched controls, these male-specific sleep and activity alterations parallel deficits seen in human ASD and ADHD patients. Additionally, while no systematic analysis of sleep has been performed in a population of human 16p11.2 hemideletion patients, sleep disturbances have been reported in two 16p11.2 hemideletion and two 16p11.2 duplication patients (Fernandez et al., 2009; Tabet et al., 2012). To our knowledge, the present study is the first study showing male-specific sleep deficits in a rodent genetic model of neurodevelopmental disorders. These findings suggest that 16p11.2 del/+ mice are an appropriate genetic model for investigating treatment strategies and potential mechanisms underlying sleep problems, hyperactivity, and sex differences found commonly in human ASD and ADHD patients.

## **Materials and Methods**

### *Animals*

16p11.2 del/+ male mice on a mixed C57BL/6J and 129S1/SvImJ background purchased from The Jackson Laboratory (Stock #013128) were bred with females on a mixed C57BL/6J and 129S1/SvImJ background (Stock #101043). All available pups were used for experiments, no litters were culled, and all cages were fitted with Nestlets (Ancare, Bellmore, NY) for enrichment. Mice were weaned at 3 weeks of age and

remained group housed with sex-matched littermates (4-5 mice/cage) until experimentation. Adult (2.5-4.5 month old) male and female 16p11.2 del/+ and WT littermate offspring were used for all experiments in accordance with age ranges utilized in previous mouse sleep experiments (Vassalli et al., 2013; Wimmer et al., 2013; Mang et al., 2016) and guidelines for assessment of behavior in adult mice (Crawley, 2007). The age distribution was consistent between the 4 experimental groups (mean  $\pm$  SEM; Male WT:  $117.3 \pm 3.9$  days, Male 16p11.2 del/+:  $115.7 \pm 3.9$  days, Female WT:  $118.2 \pm 4.6$  days, Female 16p11.2 del/+:  $117.5 \pm 4.1$  days). Separate cohorts of mice were used for all behavioral experiments. Animals were provided food and water *ad libitum* and maintained on a 12 hour light / 12 hour dark cycle with light onset at 7:00 am. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and conducted in accordance with the National Institutes of Health guidelines.

### *Activity Monitoring*

Activity was monitored using an infrared beam-break system (Opto M3, Columbus Instruments, Columbus, OH), which provided a scaffold of high-resolution infrared lights and detectors. The beams were spaced 0.5 inches apart and beam breaks were sampled every 10 seconds. Two infrared grids at 0.75 inches and 2.75 inches from the cage floor measured horizontal and vertical (rearing) activity, respectively. Mice were housed individually in noise-attenuating chambers (22"  $\times$  16"  $\times$  19", Med Associates, St. Albans, VT) equipped with individual lights (250 lux) and fans. Cages were placed within the beam break system and covered with a lid to contain the mouse and to reduce brightness (80 lux at cage floor level). To mitigate the potential impact of anxiety, mice were allowed to

acclimate to the lighting and social isolation of the activity chambers for 1 week before experimentation. Following 1 week of acclimation, activity data was collected for 1 week under 12-hour light / 12-hour dark conditions. Beam break counts were binned into 1 hour bins and averaged over the 1 week of data collection. Lighting conditions were then switched to 24-hour constant darkness for 2 weeks and counts of beam breaks were compiled every 1 minute. Circadian period ( $\tau$ ) was calculated from Day 2 to Day 14 of constant darkness using ClockLab software (Actimetrics). Because of the circadian manipulations of activity experiments, and to ensure consistent age ranges between sleep and activity experiments, separate cohorts of mice were used for activity monitoring and polysomnography sleep experiments.

### *Polysomnography*

Animals were surgically implanted with electroencephalography (EEG) and electromyography (EMG) electrodes under isoflurane anesthesia as described previously (Wimmer et al., 2013). Briefly, electrodes consisted of Teflon-coated wires (Cooner wires, Chatsworth, CA) soldered to gold socket contacts (Plastics One, Roanoke, VA) and pushed into a six-pin plastic plug (MS363 plug, Plastics One). Electrodes were held in place with miniature screws (J.I. Morris Co, Southbridge, MA) and dental cement (Ketac, 3M, St Paul, MN). Mice were allowed to recover from surgery for a minimum of 2 weeks. During the second week of recovery, animals were connected to amplifiers using lightweight cables (363, Plastics One) attached to a rotating commutator (SLC6, Plastics One). Mice were allowed to acclimate to the cables and to the noise-attenuating faraday recording chambers (38" x 39" x 33", Med Associates, Georgia, VT) for one week before analysis of sleep/wake. All recordings were obtained using parietal (ML  $\pm$  1.5 mm, AP -2

mm from bregma) electrodes referenced to an electrode over the cerebellum (-1.5 mm from lambda). Cerebellar reference was chosen based upon a lack of signal disruption from either neck muscles or other brain areas, and has been previously validated by our lab (Wimmer et al., 2013) and others (McShane et al., 2010; Vassalli et al., 2013).

EEG/EMG signals were sampled at 256 Hertz (Hz) and filtered at 0.3-30 Hz and 1-100 Hz, respectively, with 12A5 amplifiers (Astro-Med, West Warwick, RI). Data acquisition and manual visual scoring was performed using SleepSign software (Kissei Comtec Inc, Japan). EEG/EMG data was collected for a total of 24 consecutive undisturbed hours beginning at 7:00am (onset of the light phase). EEG/EMG data was analyzed in 4 second epochs as wake, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep by a trained experimenter blind to experimental conditions. Wake was classified by increases in higher frequency waves (>10 Hz), decreases in amplitude of the EEG, and high movement in the EMG. NREM was classified by increases in delta power (0.5-4 Hz) and amplitude of the EEG, along with low amplitude activity in the EMG. REM was determined by high EEG activity in the theta range (4-8 Hz) and very low activity in the EMG. One 16p11.2 del/+ male was excluded from all analyses because its total wake time over the 24-hour period was 236 minutes less than the group average, >2.5 standard deviations below the group mean. For EEG spectral analysis, fast Fourier transform (FFT; Hanning window; 0.5-20 Hz, 0.25 Hz resolution) was performed across the 24-hour period on artifact-free epochs, and wake, NREM, and REM were normalized and expressed as a percentage of the total spectral power. For a subset of the animals (15 males and 5 females), spectral analysis was not appropriate due to technical error with amplifier settings in initial cohorts and/or the quality of the EEG traces.

### *Bout Distributions*

Bouts of wake, NREM, and REM were classified as reported previously (Watson et al., 2015). Briefly, wake and NREM bouts were identified as 32 seconds (8 epochs) or greater of consecutive wake or NREM, respectively, while bouts of REM were identified as 20 seconds (5 epochs) or greater of consecutive REM. Bouts of NREM and REM were considered broken once interrupted by 8 epochs of any other state, while bouts of wake were terminated by a single epoch of any other state. Bouts of wake, NREM, and REM were then sorted into duration bins following a  $\log_2$  pattern (30-80 s, 84-160 s, 164-320 s, 324-640 s, 644-1280 s, 1284-2560 s, and >2560 s) in accordance with previously published analysis (Mochizuki et al., 2004; Kantor et al., 2013). One WT male was excluded from all analyses due to a bout of >7 hours of consecutive wakefulness, >2.5 standard deviations above the group average.

### *Elevated Zero-maze*

Mice were individually placed on a 5.5 cm wide circular track with an external diameter measuring 45 cm, raised 40 cm above the floor (San Diego Instruments, San Diego, CA). The track had two open and two enclosed segments of equal dimensions. Mice were gently placed in the center of a closed segment to begin a 5 minute trial. Time spent in open arms, risk assessment (Karlsson et al., 2005), and total distance traveled were assessed using an automated MATLAB (Mathworks) based image analysis software as described previously (Patel et al., 2014).

### *Open field*

Spontaneous activity was assessed in an open field arena (14" x 14", San Diego Instruments, San Diego, CA) fitted with photocells to detect motion. Mice were gently placed individually into the center of the open field for a 10 minute trial. Total ambulation, center activity, periphery activity, and rearing behavior were recorded. Each trial was digitally recorded and analyzed with image analysis software (Mathworks). Separate cohorts of mice were used for elevated zero-maze and open field tasks.

### *Statistics*

All statistical analyses were performed using SPSS for Windows (V. 22.0). For beam break activity, Mixed Design ANOVAs were used with genotype (WT or 16p11.2 del/+) and sex (male or female) as the between-subjects factors and time as the within-subjects factor. Two-way ANOVAs were used to compare wake, NREM and REM sleep times averaged over 24 hours, and to analyze elevated zero-maze and open field data. For 4x6-hour binned sleep state analysis, sleep state bout distribution experiments, and FFT spectral analysis, Mixed Design ANOVAs were used with genotype (WT or 16p11.2 del/+) as the between-subjects factor and time (or in the case of FFT analysis, frequency bin) as the within-subjects factor. Post hoc multiple comparisons were performed using Bonferroni's adjustment for multiple comparisons. In instances where the assumption of sphericity was violated, Greenhouse-Geisser corrected F values are given. Mann-Whitney U-tests were performed on normalized FFT data binned into low delta, delta, theta, alpha, and beta frequency bins. Multivariate analysis of variance (MANOVA) was used on the proportion of time spent in each sleep state during the light phase and the dark phase, with alpha corrected for multiple ANOVAs and set at  $\alpha = 0.05/2$ , followed by post hoc Bonferroni's adjustment for multiple comparisons.

## Results

### *Home-cage hyperactivity in 16p11.2 del/+ mice*

Male and female 16p11.2 del/+ mice and WT littermates were acclimated and assessed for home-cage activity across the diurnal cycle by breaks of infrared beams in the horizontal axis (Fig. 2.1a) as well as the vertical axis (Fig. 2.1b) to measure rearing behavior. Mixed Design ANOVAs revealed that 16p11.2 del/+ mice had significantly more activity than WT mice in both the horizontal (main effect of genotype;  $F(1,48) = 27.808$ ,  $p < 0.001$ , Fig. 2.1c) and vertical axis (main effect of genotype;  $F(1,48) = 34.714$ ,  $p < 0.001$ , Fig. 2.1d). There was no effect of sex ( $F(1,48) = 0.814$ ,  $p = 0.371$ ) nor a sex\*genotype interaction ( $F(1,48) = 0.083$ ,  $p = 0.774$ ). To test whether the hyperactive behavior observed in 16p11.2 del/+ mice may be related to stress/anxiety, we performed elevated zero-maze on a separate cohort of 16p11.2 del/+ and WT mice. There were no significant differences in time spent in the open arm (Two-way ANOVA,  $p = 0.279$ , Fig. 2.1e) or risk assessment behavior (Karlsson et al., 2005) ( $p = 0.54$ , Fig. 2.1f), demonstrating that 16p11.2 del/+ mice display normal anxiety-like behavior. There were also no differences between 16p11.2 del/+ mice and WT mice in distance traveled (Two-way ANOVA,  $p = 0.404$ , Fig. 2.1g) or time spent in the center ( $p = 0.501$ , Fig. 2.1h) in a 10 minute novel open field task.

Because the hyperactivity observed in 16p11.2 del/+ males and females was most pronounced during the dark (active) phase, and circadian problems such as increased latency to sleep and early night awakenings are commonly reported in ASD patients (Glickman, 2010b), we tested whether 16p11.2 del/+ mice have altered free-running

circadian rhythms. Male and female 16p11.2 del/+ mice were entrained to constant darkness for 2 weeks and activity was quantified via infrared beam breaks. There were no effects of genotype on circadian period ( $\tau$ ) in male (WT:  $23.70 \pm 0.04$  hrs, 16p11.2 del/+:  $23.76 \pm 0.05$  hrs;  $p = 0.36$ ) or female (WT:  $23.57 \pm 0.05$  hrs, 16p11.2 del/+:  $23.64 \pm 0.02$  hrs;  $p = 0.22$ ) mice. This indicates that 16p11.2 del/+ mice have normal circadian rhythms and also that the hyperactivity observed in these mice is intrinsic and not a product of environmental light cues.

### ***Male-specific sleep decrements in 16p11.2 del/+ mice***

Because 16p11.2 del/+ male and female mice exhibited robust hyperactivity, we assessed sleep and wake in 16p11.2 del/+ male and female mice using polysomnography recordings to distinguish between activity and sleep. EEG/EMG was recorded and assessed across a 24-hour period. Female mice were awake significantly more than male mice across the 24-hour day (main effect of sex;  $F(1,51) = 6.318$ ,  $p = 0.015$ ) consistent with previously published results showing sex differences in sleep time and architecture between male and female mice (Koehl et al., 2006; Paul et al., 2006). There was no main effect of genotype ( $F(1,51) = 2.628$ ,  $p = 0.111$ ) nor sex\*genotype interaction ( $F(1,51) = 2.110$ ,  $p = 0.152$ ). Because of the inherent sleep differences between male and female mice and the main effect of sex within our dataset, male and female 16p11.2 del/+ mice were analyzed separately and compared against sex-matched WT littermates in order to focus on the biologically relevant comparisons. Male 16p11.2 del/+ mice exhibited more wake across the 24-hour day compared to sex-matched WT mice (Student's t-test,  $p = 0.008$ ; Fig. 2.2a). There was a significant difference in 16p11.2 del/+ male wake time compared to WT males across the light/dark cycle (MANOVA,  $F(2,27) = 4.628$ ,  $p = 0.019$ ; Fig.



2.2a). *Post hoc* analysis revealed that 16p11.2 del/+ males have significantly more wake time during the light phase ( $F(1,28) = 5.322$ ,  $p = 0.029$ ), but not during the dark (active) phase ( $F(1,28) = 2.780$ ,  $p = 0.107$ ) than WT males. Concordant with increased total wake time, 16p11.2 del/+ male mice have less NREM sleep time than WT mice across the 24-hour period (Student's t-test,  $p = 0.013$ ; Fig. 2.2b). In contrast to differences in total wake and NREM time, there was no difference in REM time in 16p11.2 del/+ males relative to WT mice (Student's t-test,  $p = 0.94$ ; Fig. 2.2c). Sleep data was next analyzed in 6 hour time bins as previously published (Franken et al., 1999). Mixed Design ANOVA revealed that the decreased NREM time observed in 16p11.2 del/+ males (main effect of genotype;  $F(1,28) = 7.019$ ,  $p = 0.013$ ; Fig. 2.2d) was due primarily to the final 6 hours of the light phase (Student's t-test,  $t(28) = 9.055$ ,  $p = 0.005$ ; Fig. 2.2d). In contrast to the males, 16p11.2 del/+ females exhibited no differences in wake (Student's t-test,  $p = 0.92$ ; Fig. 2.2e), NREM (Student's t-test,  $p = 0.53$ ; Fig. 2.2f), or REM (Student's t-test,  $p = 0.067$ ; Fig. 2.2g) time compared to WT females across the 24-hour period. Female 16p11.2 del/+ mice also exhibited no differences in wake, NREM, or REM in the light or dark phases (wake:  $F(2,22) = 0.414$ ,  $p = 0.67$ , Fig 2.2e; NREM:  $F(2,22) = 1.633$ ,  $p = 0.218$ , Fig 2.2f; REM:  $F(2,22) = 3.121$ ,  $p = 0.064$ , Fig 2.2g). Analyzing female 16p11.2 del/+ NREM sleep in 6 hour time bins revealed no significant main effect ( $F(3,69) = 0.397$ ,  $p = 0.535$ ; Fig. 2h) nor a significant genotype\*time interaction ( $F(3,69) = 1.272$ ,  $p = 0.29$ ; Fig. 2.2h). This data suggests that 16p11.2 del/+ males, but not females, have deficits in either sleep initiation or sleep maintenance.

### ***16p11.2 del/+ males have elongated bouts of wakefulness***

Because 16p11.2 del/+ male mice have decreased sleep and increased wakefulness, we binned and analyzed the distribution of wake, NREM, and REM bout durations as described previously (Mochizuki et al., 2004; Kantor et al., 2013) to elucidate the factor(s) contributing most strongly to this phenotype. We found that 16p11.2 del/+ male mice have an altered distribution of wake bout length duration (genotype\*bout time interaction;  $F(6,168) = 6.599$ ,  $p = 0.001$ ; Fig. 2.3a). *Post hoc* comparisons indicated that 16p11.2 del/+ males spent a higher proportion of their wake time in prolonged bouts of continuous wakefulness (>42 consecutive minutes:  $t(28) = 11.340$ ,  $p = 0.002$ ) and less time in bouts of shorter duration (160-320 seconds:  $t(28) = 5.356$ ,  $p = 0.028$ ; 320-640 seconds:  $t(28) = 10.248$ ,  $p = 0.003$ ; 640-1280 seconds:  $t(28) = 6.480$ ,  $p = 0.017$ ). By contrast, females exhibited no differences in wake bout length distribution ( $F(6,138) = 1.576$ ,  $p = 0.22$ ; Fig. 2.3d). There were no differences in NREM bout time distribution in 16p11.2 del/+ males ( $F(6,168) = 0.500$ ,  $p = 0.59$ ; Fig. 2.3b) or females ( $F(6,138) = 1.924$ ,  $p = 0.16$ ; Fig. 2.3e). Likewise, there were no differences in REM bout length distribution in either males ( $F(6,168) = 0.785$ ,  $p = 0.48$ ; Fig. 2.3c) or females ( $F(6,138) = 1.816$ ,  $p = 0.16$ ; Fig. 2.3f). Together, this data indicates that 16p11.2 del/+ males, but not females, sleep less than WT mice due to deficits in sleep initiation, rather than sleep maintenance.

### ***16p11.2 del/+ males have reduced alpha power during wake***

Next, we performed fast Fourier transform (FFT) to analyze the EEG power spectra of 16p11.2 del/+ males and females during wake, NREM, and REM. There was no main effect of genotype for any sleep state for either sex (Males wake:  $F(1,13) = 2.649$ ,  $p = 0.13$ , Fig. 2.4a; NREM:  $F(1,13) = 3.851$ ,  $p = 0.07$ , Fig. 2.4b; REM:  $F(1,13) = 0.760$ ,  $p = 0.40$ , Fig. 2.4c; Females wake:  $F(1,18) = 0.649$ ,  $p = 0.43$ , Fig. 2.4d; NREM:  $F(1,18) =$

1.680,  $p = 0.21$ , Fig. 2.4e; REM:  $F(1,18) = 0.744$ ,  $p = 0.40$ , Fig. 2.4f). However, binning the data into low delta (0.5-1.5 Hz), delta (0.5-4.0 Hz), theta (4.0-8.0 Hz), alpha (8.0-12.0 Hz), and beta (12.0-20.0 Hz) frequency bands revealed that 16p11.2 del/+ males have significantly increased alpha power during wake relative to WT littermates (Mann-Whitney  $U = 8$ ,  $p = 0.021$ ; Fig. 2.4a, inset), suggesting increased arousal and vigilance during quiet wake in 16p11.2 del/+ males (Cantero et al., 2002). Increased arousal during quiet wake, in concert with increases in total wake time and prolonged bouts of continuous wakefulness, suggests that 16p11.2 del/+ males may possibly have deficits initiating wake-to-sleep transitions. In contrast, there were no differences in alpha power during wake between 16p11.2 del/+ females and WT littermates (Mann-Whitney  $U = 31$ ,  $p = 0.18$ , Fig. 2.4d, inset). 16p11.2 del/+ females, however, had significantly increased beta power during wake (Mann-Whitney  $U = 22$ ,  $p = 0.038$ , Fig. 2.4d, inset). There were no differences in any other frequency bands apart from a decrease in low delta power during NREM in 16p11.2 del/+ females in comparison to WT littermates (Mann-Whitney  $U = 16$ ,  $p = 0.010$ , Fig. 2.4e, inset), which may indicate differences in sleep homeostasis.

## Discussion

We investigated home-cage activity and sleep patterns in one mouse model of human 16p11.2 chromosomal hemideletion. We report robust and reliable home-cage hyperactivity across the light-dark cycle that is present in both males and females. These findings expand upon previous reports of hyperactivity in 16p11.2 del/+ mice (Horev et al., 2011; Brunner et al., 2015; Arbogast et al., 2016) by comparing males and females, assessing activity in the home-cage, and quantifying activity over a week of consecutive diurnal cycles. Importantly, there are presently three distinct mouse models of 16p11.2

hemideletion differing in deletion size and genetic background, which should be considered when comparing findings between studies. In addition, we report the first male-specific sleep decrements in a rodent model of ASD. Decreased total sleep time and prolonged bouts off wakefulness, suggesting difficulties in initiating wake-to-sleep transition, recapitulate common sleep and activity problems reported in human ASD patients (Miano et al., 2007; Krakowiak et al., 2008; Reynolds & Malow, 2011; Baker & Richdale, 2015). The sleep/wake deficits in 16p11.2 del/+ males, but not females relative to sex-matched controls support theories of female protectiveness from ASD given the same genetic insult (Robinson et al., 2013; Werling & Geschwind, 2013; Jacquemont et al., 2014).

Additionally, compared to sex-matched controls, the sleep/wake differences observed only in male 16p11.2 del/+ mice are contrasted by activity differences in both 16p11.2 del/+ males and females. This distinction suggests disparate neurobiological mechanisms underlying the two alterations. The etiology of sleep and activity deficits in ASDs remains unknown, but numerous lines of evidence strongly support a relationship between imbalanced excitatory/inhibitory signaling, sleep, and hyperactivity. Although both ADHD and ASD are associated with imbalanced excitatory/inhibitory signaling, the neurochemical and neuroanatomical mechanisms mediating these disorders are likely distinct. The sex differences in sleep/wake, but not activity in 16p11.2 del/+ mice therefore suggest that the 16p11.2 del/+ mouse model may be a useful rodent genetic model for investigating neurobiological mechanisms mediating sex differences in neurodevelopmental disorders.

### ***Neurochemical and neuroanatomical correlates in 16p11.2 hemideletion***

The mechanisms underlying altered sleep and activity in our 16p11.2 del/+ mouse model may be considered in light of recent neurochemical and neuroanatomical findings in related models. Another mouse model of 16p11.2 hemideletion was recently shown to have increased GABAergic medium spiny neurons (MSNs) expressing the dopamine D2 receptor in the striatum, and decreased dopamine D1 receptor neurons in the cortex (Portmann et al., 2014). During development, D1 activation increases and D2 activation decreases GABA neuron migration from the basal forebrain to the cortex (Crandall et al., 2007). Further, estrogen in females has been found to downregulate D2 receptor function (Bazzett & Becker, 1994). In *Drosophila*, knock down of the E3 ubiquitin ligase Cul3, which interacts with the 16p11.2 region gene KCTD13 through the KCTD13-Cul3-RhoA pathway, results in increased dopamine signaling, reduced sleep duration, and hyperarousal (Stavropoulos & Young, 2011; Pfeifferberger & Allada, 2012), similar to our findings in 16p11.2 del/+ mice. Hyperactivity is often thought of as a disorder of catecholamine dysfunction (Sharma & Couture, 2014), and the striatal dopaminergic system has a strong wake-promoting role (Saper et al., 2010). Together, these findings suggest that dysregulated dopaminergic signaling may underlie some of the sleep and activity alterations observed in 16p11.2 del/+ mice.

Imaging studies of humans and mice with 16p11.2 hemideletion reveal alterations in structure and connectivity of the striatum and frontal cortex. Magnetic resonance imaging (MRI) studies show increased striatal volume and cortical surface area in 16p11.2 hemideletion humans (Qureshi et al., 2014) and mice (Portmann et al., 2014), agreeing with structural changes reported more broadly in a meta-analysis of ASD patients (Nickl-Jockschat et al., 2012). 16p11.2 hemideletion children show widespread white matter

abnormalities, including increased axial diffusivity of the corpus callosum, external capsule, and internal capsule—the last of which is comprised largely of the corticospinal tract, carrying projections from the primary motor cortex through the striatum to the spinal cord (Owen et al., 2014). Using diffusion tensor imaging (DTI), we have shown male-specific white matter alterations in the same fiber bundles proximal to the striatum in 16p11.2 del/+ mice (Grissom et al., 2017; Nickl-Jockschat et al., 2015). The striatum and frontal cortex are important brain regions mediating motor control and sleep/wake. Hyperactive behavior is generally believed to relate to abnormalities in corticostriatal connectivity (Bush, 2010), and children with ASD show reduced striatal activation in response to rewards (Scott-Van Zeeland et al., 2010; Kohls et al., 2013). Striatal growth rate is also correlated with ASD diagnosis (Langen et al., 2014). Together, these results may implicate corticostriatal deficits in the hyperactive behavior and increased wakefulness observed in 16p11.2 hemideletion mice.

### ***Conclusions and future directions***

Sleep and activity problems are among the most common complaints reported in neurodevelopmental disorders. Psychomotor stimulants are often the first-choice option for treating hyperactivity, but typically exacerbate sleep problems in ADHD patients (Lee et al., 2012; Santisteban et al., 2014). Nocturnal melatonin, which is decreased in insomniacs (Rodenbeck et al., 1999) and children diagnosed with ASD (Tordjman et al., 2005), has been effective for decreasing sleep latency and increasing total sleep time in some studies of ASD patients (Wright et al., 2011), but has little effect on mitigating other sleep disturbances (Cortesi et al., 2012; Malow et al., 2012) and may even increase night awakenings (Rossignol & Frye, 2011; Gringras et al., 2012). Unraveling the distinct

neurochemical mechanisms mediating the sleep and hyperactivity phenotypes observed in 16p11.2 hemideletion mice is an interesting avenue of investigation that may lead to a greater understanding of the behavioral and cognitive deficits observed in 16p11.2 hemideletion patients, as well as signaling mechanisms contributing to sex-biased ASD symptomology. Male-specific neuroanatomical and neurochemical differences in 16p11.2 del/+ mice, such as increased white matter proximal to the striatum (Grissom et al., 2017), striatal hypertrophy (Portmann et al., 2014), and possibly increased D2 receptor expression and activity of striatal MSNs may contribute to the sex-specific differences in sleep. Conversely, neurochemical and neuroanatomical deficits shared between 16p11.2 del/+ males and females, such as decreased ERK1 protein in the striatum, or decreased white matter integrity of the corpus callosum (Grissom et al., 2017), may underlie the hyperactivity observed in both 16p11.2 del/+ males and females. Future dose-response studies of changes in sleep and activity in 16p11.2 del/+ mice in response to dopamine receptor subtype-specific drugs may reveal the contributions of these receptors and signaling pathways to the insomnia and hyperactivity observed in 16p11.2 del/+ male mice. These experiments are the first steps in elucidating the signaling mechanisms involved in 16p11.2 hemideletion hyperactivity and insomnia, and may lead to more targeted brain-region or cell-type specific experiments, as well as a more complete understanding of the neurochemical mechanisms mediating behavior in a common CNV found in ASDs. Together, these findings demonstrate that the 16p11.2 del/+ mouse model will be useful for investigating the molecular basis of sex bias in ASDs, as well as distinct neural mechanisms underlying common sleep and activity problems in ASDs and related neurodevelopmental disorders.

**Author Contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Thomas Nickl-Jockschat and Ted Abel. Christopher Angelakos and Ted Abel designed the study. Christopher Angelakos, Timothy O'Brien, and Kyle Krainock conducted behavioral experiments. Christopher Angelakos analyzed activity and polysomnography behavioral data. Adam Watson programmed and analyzed bout distribution data.

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## Figure Legends

**Figure 2.1. 16p11.2 del/+ mice are hyperactive throughout the diurnal cycle. A,B)** Infrared beam breaks in the XY (horizontal) (A) and Z (vertical) (B) axis plotted for all four groups, in 1 hour bins, across the 24 hour day. Gray box indicates the dark (active) period. **C,D)** 16p11.2 del/+ mice have significantly greater activity relative to wildtype littermates, irrespective of sex, in both the horizontal (C) and vertical (D) axes. **E,F)** There are no significant differences between 16p11.2 del/+ and wildtype mice in elevated zero-maze time spent in open arms (E) or risk assessment (F). **G,H)** There are no differences in open field total ambulation (G) or time spent in center (H) between 16p11.2 del/+ mice and wildtype controls. Mean  $\pm$  standard error of the mean (s.e.m.) \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

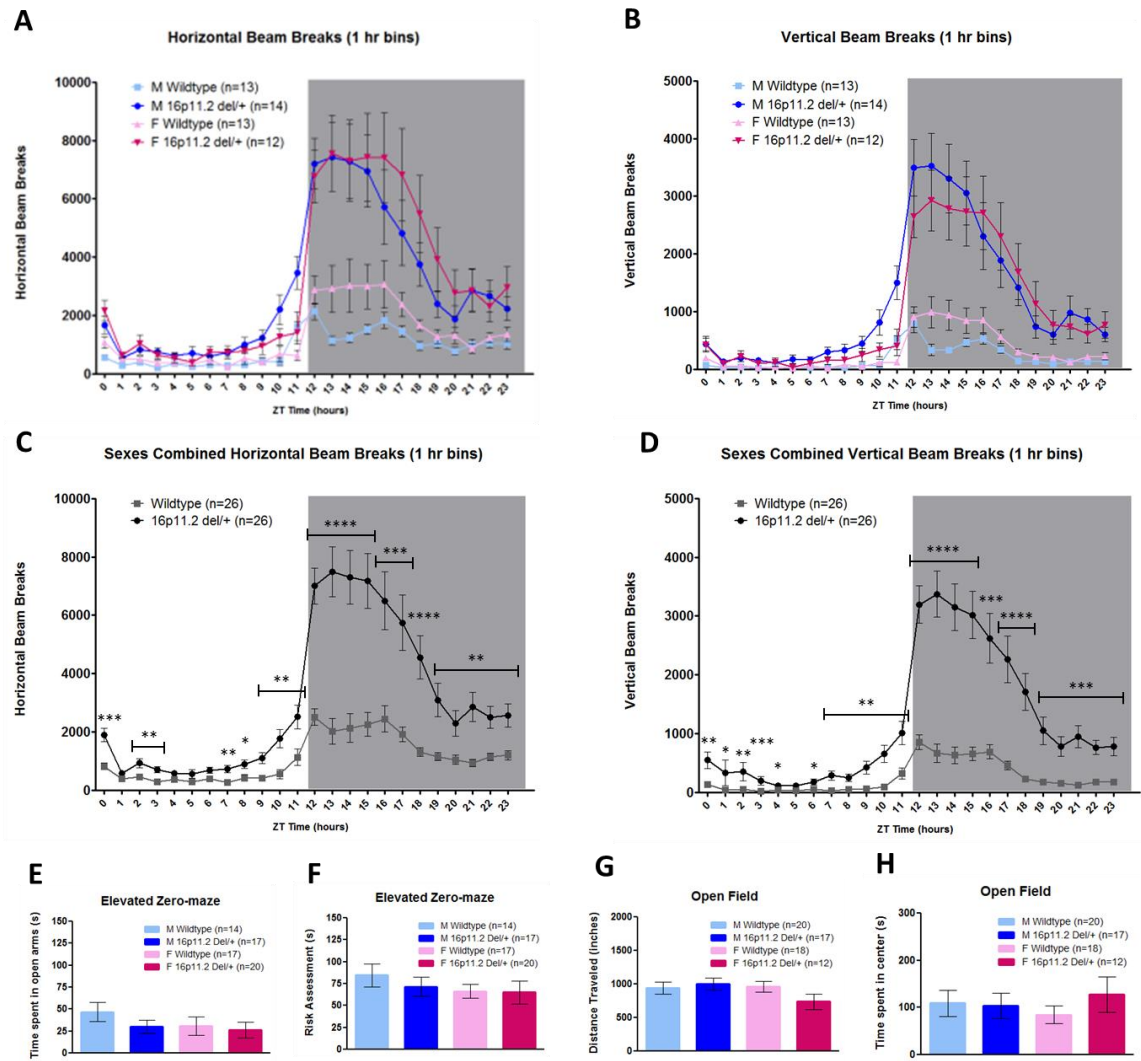
**Figure 2.2. Male 16p11.2 del/+ mice sleep less than wildtype littermates. A-C)** Male 16p11.2 del/+ mice are awake significantly more (A) and spend significantly less time in NREM sleep (B) than wildtype mice over the 24-hour period. The amount of REM sleep in male 16p11.2 del/+ mice was not significantly different from wildtype (C). **D)** NREM sleep time expressed in 6 hour bins reveals that males have significantly less sleep during the last 6 hours of the light in comparison to wildtype littermates. Gray box indicates the dark period. **E-F)** There are no significant differences in wake (E), NREM sleep (F,H), or REM sleep (G) time between 16p11.2 del/+ females and controls. Mean  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.01$

**Figure 2.3. Male 16p11.2 del/+ mice spend a significantly higher proportion of wake time in prolonged bouts of wakefulness.** Proportion of total wake time across the 24-hour period divided into bout lengths of 30s-80s, 80s-160s, 160s-320s, 320s-640s, 640s-1280s, 1280s-2560s, and >2560s. **A)** 16p11.2 del/+ male mice spend a significantly greater proportion of their wake time in long bouts of wakefulness (>2560s) and a significantly lower proportion of time in

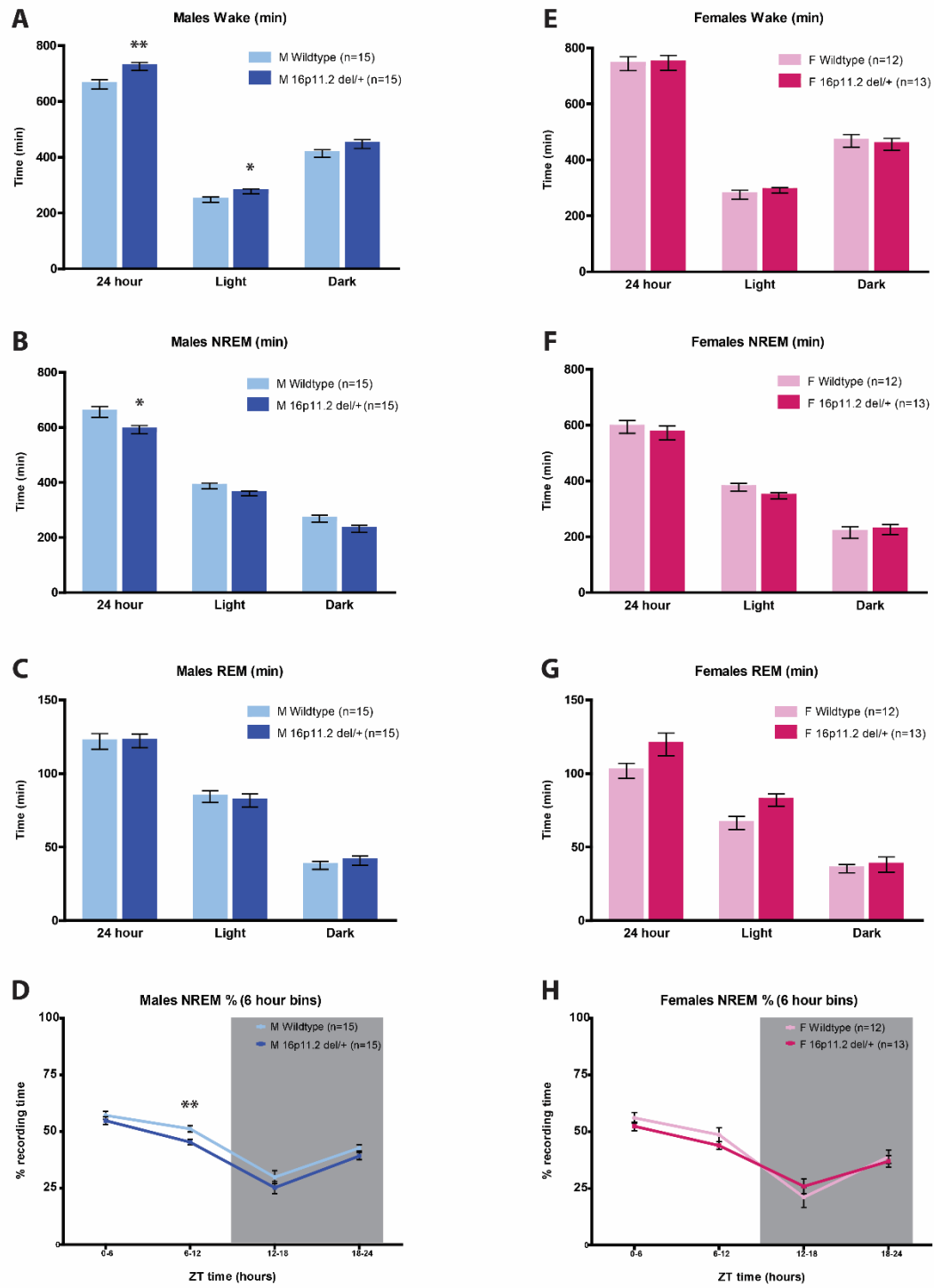
intermediate bouts of wakefulness (160s-1280s). **B-C)** 16p11.2 del/+ males exhibit no differences in NREM (B) or REM (C) bout length distribution. **D-F)** 16p11.2 del/+ female mice do not differ from wildtype littermates in distribution of wake (D), NREM (E), or REM (F) bout length. Mean  $\pm$  s.e.m. \* $p < 0.05$ , \*\* $p < 0.01$

**Figure 2.4. Male 16p11.2 del/+ mice have increased alpha power during wake. A)** Male 16p11.2 del/+ mice have increased alpha power during wake (A, inset). **B-C)** Male 16p11.2 del/+ mice have no differences in EEG power spectra during NREM sleep (B) or REM sleep (C). **D)** Female 16p11.2 del/+ mice have increased beta power during wake (D, inset). **E)** Female 16p11.2 del/+ mice have decreased low delta during NREM sleep (E, inset). **F)** Female 16p11.2 del/+ mice have no differences in EEG power spectra during REM sleep (F). Mean  $\pm$  s.e.m. \* $p < 0.05$ .

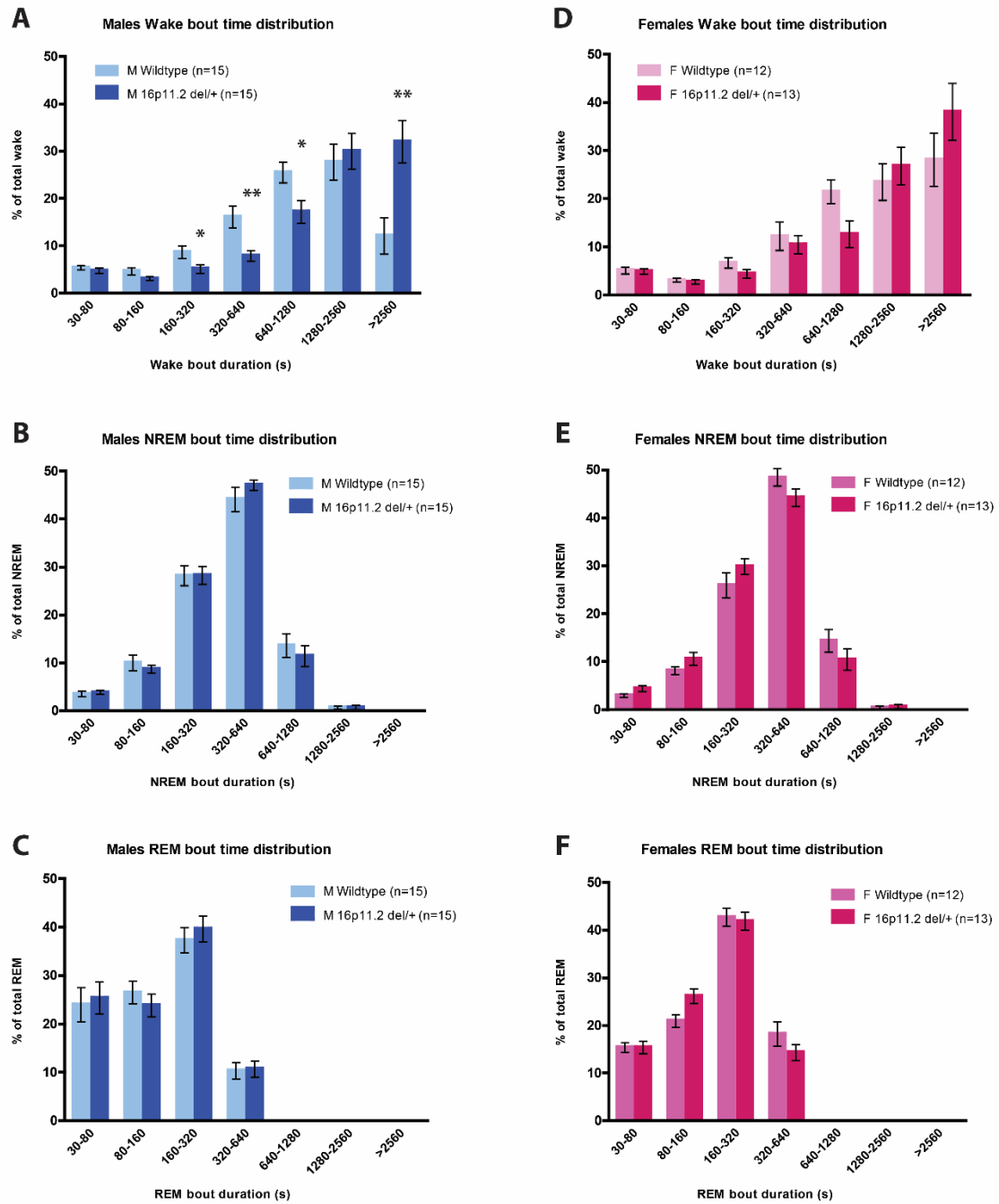
Figure 2.1



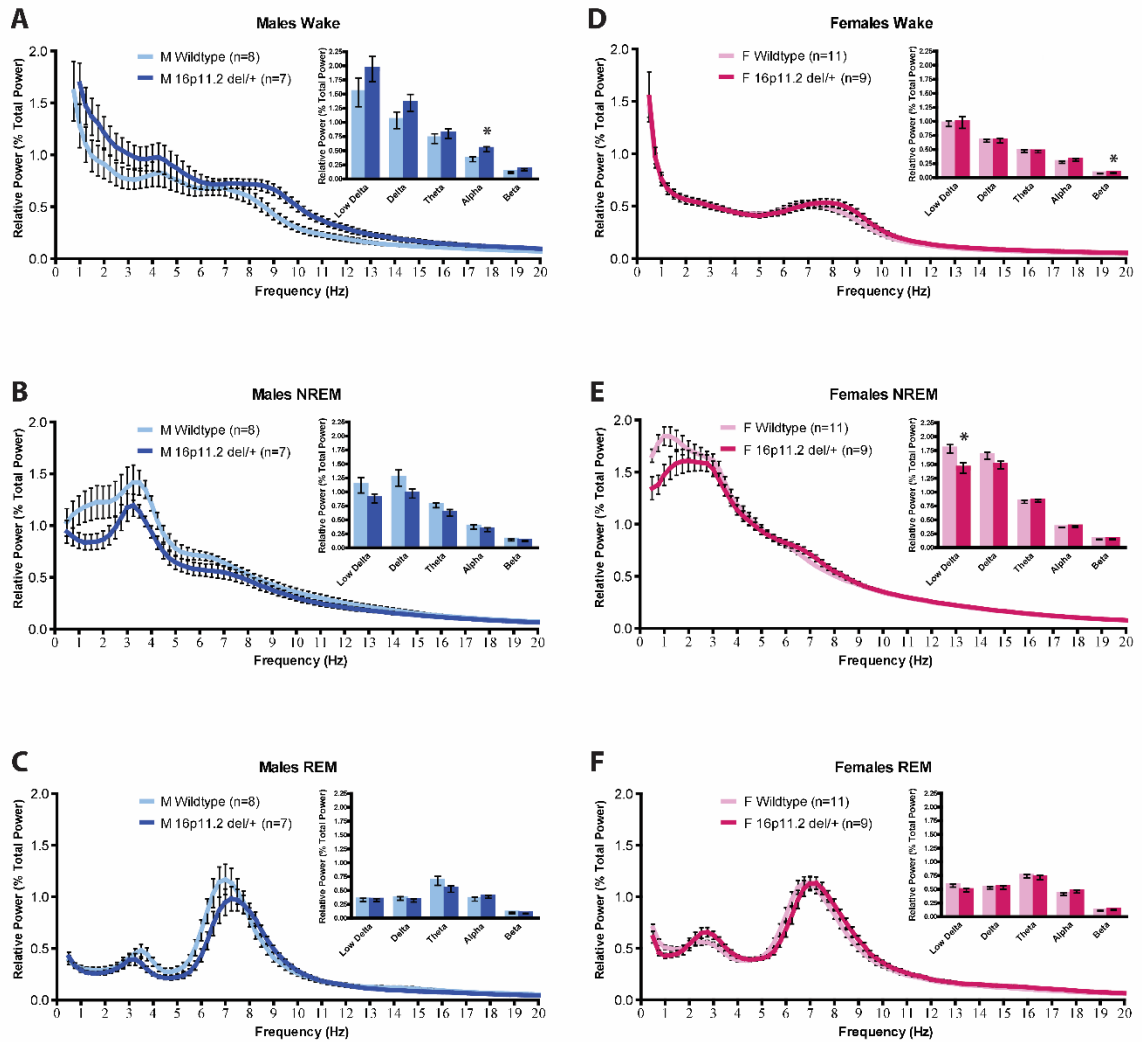
**Figure 2.2**



**Figure 2.3**



**Figure 2.4**



### CHAPTER 3: REM sleep reductions, EEG spectra alterations, and altered homeostatic sleep rebound in *Syngap1*<sup>+/-</sup> mice

#### Abstract

Sleep problems are common in neurodevelopmental disorders, reducing quality of life for patients and their caretakers. Mutations in the Synaptic Ras-GTPase-activating protein (SYNGAP1) are strongly associated with intellectual disability (ID), autism spectrum disorders (ASD), and epilepsy. Patients with SYNGAP1 mutations exhibit sleep disruptions, seizures, and several other physical, behavioral, and cognitive symptomologies. Many of these behavioral and electrophysiological issues are recapitulated in *Syngap1*<sup>+/-</sup> mice. In this study, we sought to characterize sleep/wake and EEG spectra properties in *Syngap1*<sup>+/-</sup> male mice using polysomnography. We found significantly decreased quantities of REM sleep, NREM and REM sleep bouts, and NREM to REM sleep-state transitions in *Syngap1*<sup>+/-</sup> mice relative to WT littermates. Analysis of EEG spectra revealed significantly elevated EEG delta power (0.5-4.0 Hz) in NREM sleep and significantly attenuated alpha (8.0-12.0 Hz) and beta power (12.0-20.0 Hz) during wake in *Syngap1*<sup>+/-</sup> mice compared to WT controls. Following a six-hour sleep deprivation challenge, *Syngap1*<sup>+/-</sup> mice had abnormal homeostatic sleep rebound. Together, this study identifies altered sleep and EEG spectra properties in *Syngap1*<sup>+/-</sup> mice, suggesting that *Syngap1*<sup>+/-</sup> mice may provide an accurate rodent genetic model for studying the neurobiological mechanisms underlying sleep problems in SYNGAP1 mutated patients and neurodevelopmental disorders in general.

## Introduction

Disrupted sleep is one of the most commonly reported issues in a wide range of neurodevelopmental disorders, including autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), schizophrenia, and intellectual disability (ID). Sleep issues in neurodevelopmental disorders are widespread, manifesting as insomnia, poor sleep efficiency, and circadian issues (Didden et al., 2002; Robinson & Richdale, 2004; Richdale & Schreck, 2009; Glickman, 2010; Kirov & Brand, 2014; Picchioni et al., 2014; Robinson-Shelton & Malow, 2016; Souders et al., 2017). Moreover, sleep problems are positively correlated with the severity of other symptomologies in neurodevelopmental disorders and have been estimated to attribute 22-32% of the variance in behavioral abnormalities in ASD (Park et al., 2012; Mazurek & Sohl, 2016). Systematic and well-controlled polysomnography data from children with neurodevelopmental disorders and mouse models of neurodevelopmental disorders is sparse.

Mutation of the Synaptic Ras-GTPase-activating protein (SYNGAP1) is consistently identified as one of the highest single-gene risk factors associated with neurodevelopmental disorders (Hoischen et al., 2014; Zhu et al., 2014; Deciphering Developmental Disorders Study, 2015). Humans with SYNGAP1 mutations frequently receive diagnoses of intellectual disability, autism, and epilepsy. Like many genes associated with increased prevalence for developing a neurodevelopmental disorder, SYNGAP1 is enriched in the postsynaptic density (PSD) and plays a role in excitatory/inhibitory balance. SYNGAP1 is a negative regulator of RAS-GTPase in the PSD, and reduction of SYNGAP1 results in increased ERK signaling, increased AMPA receptor trafficking to the membrane, hyperexcitability, and precocious spine development



in heterozygous mice (*Syngap1*<sup>+/-</sup> mice) (Vazquez et al., 2004; Rumbaugh et al., 2006; Clement et al., 2012; Clement et al., 2013; Aceti et al., 2015). To date, *Syngap1*<sup>+/-</sup> mice have provided both face and construct validity for numerous behaviors associated with SYNGAP1 mutation, including lower seizure threshold, cognitive deficits, stereotypies, and social abnormalities (Ozkan et al., 2014; Jeyabalan & Clement, 2016; Ogden et al., 2016).

Humans with SYNGAP1 mutation frequently report disrupted sleep (Berryer et al., 2013; M. J. Parker et al., 2015). Despite this, quantitative analyses of sleep/wake and EEG power spectra have not been performed in *Syngap1*<sup>+/-</sup> mice or humans. Given the associations between impaired sleep, neurodevelopmental disorders, and epilepsy, as well as the presence of sleep problems in SYNGAP1 mutated humans, we investigated numerous parameters of sleep and wake in *Syngap1*<sup>+/-</sup> mice. We found REM sleep reductions, altered sleep/wake state transitions, EEG spectral abnormalities, and impaired homeostatic response to sleep deprivation in *Syngap1*<sup>+/-</sup> male mice compared to WT controls. These findings suggest that the *Syngap1*<sup>+/-</sup> mouse model may be a useful genetic model for studying the neurobiological underpinnings of sleep problems in SYNGAP1 mutation and other neurodevelopmental disorders.

## **Materials and Methods**

### *Animals*

*Syngap1*<sup>+/-</sup> mice were generated as previously described (Kim et al., 2003) and bred and maintained on a BL6/B129sv/ev hybrid background at The Scripps Research Institute (Jupiter, FL). Mice were shipped to the University of Pennsylvania (Philadelphia, PA)

between 2-3 months of age. Following 7 weeks of quarantine, mice were allowed to acclimate to the rodent colony room for at least 1 week before undergoing polysomnography surgery. All mice were between 4.5 and 6 months old during baseline and recovery sleep recordings and experimental data was collected for 12 WT males and 16 *Syngap1*<sup>+/-</sup> males. Animals were provided food and water *ad libitum* and maintained on a 12 hour: 12 hour light:dark (12h:12h LD) cycle with light onset at 7:00 am, except where noted for circadian period assessment in constant darkness, which occurred after the completion of all other experiments. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania, the Institutional Animal Care and Use Committee at The Scripps Research Institute, and conducted in accordance with the National Institutes of Health guidelines.

### *Polysomnography*

Animals were surgically implanted with electroencephalography (EEG) and electromyography (EMG) electrodes under isoflurane anesthesia as described previously (Angelakos et al., 2016; Wimmer et al., 2013). Briefly, electrodes consisted of Teflon-coated wires (Cooner wires, Chatsworth, CA) soldered to gold socket contacts (Plastics One, Roanoke, VA) and pushed into a six-pin plastic plug (MS363 plug, Plastics One). Electrodes were held in place with miniature screws (J.I. Morris Co, Southbridge, MA) and dental cement (Ketac, 3M, St Paul, MN). After one week of recovery from surgery, mice were connected to amplifiers using lightweight cables (363, Plastics One) attached to a rotating commutator (SLC6, Plastics One). Mice were allowed to acclimate to the cables and to the noise-attenuating faraday recording chambers (38" x 39" x 33", Med Associates, Georgia, VT) for one week before a 24-hour undisturbed recording of baseline sleep/wake

beginning at 7:00am (light onset). All recordings were obtained using parietal ( $ML \pm 1.5$  mm, AP -2 mm from bregma) electrodes referenced to an electrode over the cerebellum (-1.5 mm from lambda).

EEG/EMG signals were sampled at 256 Hertz (Hz) and filtered at 0.3-30 Hz and 1-100 Hz, respectively, with 12A5 amplifiers (Astro-Med, West Warwick, RI). Data acquisition and manual visual scoring was performed using SleepSign software (Kissei Comtec Inc, Japan). EEG/EMG data was analyzed in 4 second epochs as wake, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep by a trained experimenter blind to experimental conditions. For EEG spectral analysis, fast Fourier transform (FFT; Hanning window; 0.5-20 Hz, 0.25 Hz resolution) was performed across the 24-hour period on artifact-free epochs, and wake, NREM, and REM were normalized and expressed as a percentage of the total wake, NREM, and REM power, respectively.

#### *Bout classifications and distributions*

Bouts of wake, NREM, and REM were classified as reported previously (Watson et al., 2015; Angelakos et al., 2016). Briefly, wake and NREM bouts were identified as 32 seconds (8 epochs) or greater of consecutive wake or NREM, respectively. Bouts of REM were identified as 20 seconds (5 epochs) or greater of consecutive REM. Bouts of NREM and REM were considered broken once interrupted by 8 epochs of any other state, while bouts of wake were terminated by a single epoch of any other state. Bouts of wake, NREM, and REM were then sorted into duration bins following a  $\log_2$  scale in accordance with previously published analysis (Mochizuki et al., 2004; Kantor et al., 2013; Angelakos et al., 2016).

### *Sleep deprivation and recovery sleep*

Following 24-hours of undisturbed baseline recording, all mice were manually sleep deprived by gentle handling for six hours starting at 7:00am (light onset), as previously described (Vecsey et al., 2009; Havekes et al., 2016; Tudor et al., 2016). The movement, behavior, and posture of mice were monitored and sleep deprivation was maintained by tapping lightly on the cage with a ruler and/or disturbing the Nestlet/bedding material when the mice appeared to be falling asleep. EEG analysis during the six hours of sleep deprivation revealed >96% efficiency sleep deprivation for both *Syngap1*<sup>+/-</sup> and WT mice, with no group differences between genotypes. Following sleep deprivation, mice were left undisturbed for 18 hours of recovery sleep, culminating at the onset of the light cycle (7:00am) the following morning.

### *Activity Monitoring*

Following the recovery sleep day, mice were transferred into a home-cage infrared beam-break activity monitoring system (Opto M3, Columbus Instruments, Columbus, OH) and individually housed inside light- and noise-attenuating chambers (22" x 16" x 19", Med Associates, St. Albans, VT) equipped with a 250 lux light source (80 lux at cage floor) and fan for ventilation. Infrared beams spaced 0.5 inches apart provided two horizontal grids of infrared beams and detectors at 0.75 inches and 2.75 inches above the cage floor, quantifying horizontal and vertical (rearing) activity, respectively. Mice were allowed to acclimate to the activity chambers for one week, ensued by one week of continuous activity monitoring, as previously described (Angelakos et al., 2016). Activity counts were compiled every 10s and binned into 1-hour bins, averaged over the seven days of activity

monitoring. After the final day of activity monitoring in 12h:12h LD, lights were switched off and one cohort of animals (n=8 WT and n=7 *Syngap1*<sup>+/-</sup>) were allowed to free-run in 24-hour constant darkness (DD) for 2 weeks, with activity counts compiled every minute. Circadian period ( $\tau$ ) was calculated from Day 2 to day 14 of DD using ClockLab software (Actimetrics).

### *Statistics*

All statistical analyses were performed using SPSS for Windows (V. 24.0). Student's T-test was used to compare 24-hour sleep/wake state time, bout counts, bout durations, transitions, activity counts, 18-hour recovery sleep time, and circadian period ( $\tau$ ) between *Syngap1*<sup>+/-</sup> mice and WT. Multivariate analysis of variance (MANOVA) was used to compare sleep state time, bout counts, bout durations, state transitions, and activity beam breaks divided by light/dark cycle, with alpha corrected for multiple ANOVAs and set at  $\alpha = 0.05/2$ , followed by post hoc Bonferroni's adjustment for multiple comparisons. Mixed Design ANOVAs were utilized to compare binned sleep/wake state time, bout counts, bout distributions, activity counts, NREM slow wave activity, recovery sleep time, and EEG spectra, with genotype (WT or *Syngap1*<sup>+/-</sup>) as the between-subjects factor and time (or in the case of EEG spectra, frequency) as the within-subjects factor. Post hoc multiple comparisons were performed using Bonferroni's adjustment for multiple comparisons. In instances where the assumption of sphericity was violated, Greenhouse-Geisser corrected F values are given. Mann-Whitney U-tests were performed on normalized FFT data binned into low delta, delta, theta, alpha, beta, and gamma frequency bins. 2-hour binned baseline and recovery delta power data was normalized to the slow wave activity (SWA) during the last four hours of the baseline light (inactive) period, as

previously described (Franken et al., 2001). Bins with less than 10 minutes of NREM sleep over the 2-hour interval were excluded from binned SWA analyses.

## Results

### ***REM sleep time and bout reductions in *Syngap1*<sup>+/-</sup> mice***

Sleep and wakefulness was quantified in *Syngap1*<sup>+/-</sup> mice and WT littermates across an undisturbed 24-hour baseline period. There were no differences in time spent awake (Student's T-test,  $p = 0.11$ ; Fig. 3.1a,b) or in NREM sleep (Student's T-test,  $p = 0.35$ , Fig. 3.1c,d) over the 24-hour day between WT and *Syngap1*<sup>+/-</sup> mice. Analysis by light/dark cycle revealed significantly less NREM sleep during the dark (active) phase in *Syngap1*<sup>+/-</sup> mice relative to WT (MANOVA,  $F(2,25) = 4.339$ ,  $p = 0.024$ ; Fig. 3.1c,d). Quantification of REM sleep time revealed significant REM sleep reductions in *Syngap1*<sup>+/-</sup> mice compared to WT controls over the 24-hour day (Student's T-Test,  $p = 0.004$ ; Fig. 3.1e,f), and during both the light and dark cycles (MANOVA,  $F(2,25) = 4.902$ ,  $p = 0.016$ ; Fig. 3.1e,f). Interestingly, reduction in REM sleep time is commonly reported in polysomnography studies of human ASD patients (Limoges et al., 2005; Buckley et al., 2010).

Next, we quantified the number of wake, NREM, and REM bouts and the distribution of their durations in *Syngap1*<sup>+/-</sup> and WT mice. There were no differences in the number of wake bouts ( $p = 0.23$ ; Fig. 3.2a), or the distribution of their durations ( $p = 0.33$ ; Fig. 3.2b) between *Syngap1*<sup>+/-</sup> and WT mice. Analysis of NREM bout counts revealed significant reductions in the number of NREM bouts across the 24-day (Student's T-test,  $p = 0.009$ ; Fig. 3.2c) and in both the light and dark cycles (MANOVA,  $F(2,25) = 5.595$ ,  $p =$

0.10; Fig. 3.2c). Analyzing the distribution of NREM bout durations indicated reduced quantities of the shortest duration NREM bouts (30-80s and 80-160s in duration) in *Syngap1<sup>+/-</sup>* mice compared to WT ( $F(1,26) = 7.027$ ,  $p = 0.013$ ; Fig. 3.2d). Similarly, *Syngap1<sup>+/-</sup>* mice displayed substantially less REM bouts than WT mice over the 24-hour day (Student's T-test,  $p = 0.007$ ; Fig. 3.2e) and in both the light and dark phases (MANOVA,  $F(2,25) = 6.337$ ,  $p = 0.006$ ; Fig. 3.2e). Analysis of the distribution of REM bouts of varying durations revealed that *Syngap1<sup>+/-</sup>* mice have significantly less REM bouts of the shortest duration (20-40s) than WT mice ( $F(1,26) = 10.111$ ,  $p = 0.004$ ; Fig. 3.2f). There were no significant differences in mean bout duration for any sleep/wake state between *Syngap1<sup>+/-</sup>* mice and WT (Supplementary Figure 3.1).

#### ***Syngap1<sup>+/-</sup> mice have less NREM to REM transitions than WT controls***

Because of the alterations of REM sleep time and NREM and REM sleep bouts in *Syngap1<sup>+/-</sup>* mice compared to WT, we next investigated the transitions between sleep/wake states in *Syngap1<sup>+/-</sup>* and WT mice. Unsurprisingly, *Syngap1<sup>+/-</sup>* mice displayed significantly less NREM to REM transitions than WT mice over the 24-hour recording (Student's T-test,  $p = 0.005$ ; Fig. 3.3a) and during both the light and dark phases (MANOVA,  $F(2,25) = 5.816$ ,  $p = 0.008$ ; Fig. 3.3a). There were no differences in the number of NREM to wake transitions ( $p = 0.51$ ; Fig. 3.3b), Wake to NREM transitions ( $p = 0.21$ ; Fig. 3.3c), or REM to wake transitions ( $p = 0.25$ , Fig. 3.3d) between *Syngap1<sup>+/-</sup>* mice and WT.

#### ***Syngap1<sup>+/-</sup> mice have elevated delta power in NREM sleep and decreased alpha and beta power in wake in comparison to WT mice***

SYNGAP1 mutation alters excitatory/inhibitory balance by increasing AMPA receptor trafficking to the postsynaptic membrane, altering NMDA:AMPA ratio, and modifying spine development (Vazquez et al., 2004; Rumbaugh et al., 2006; Clement et al., 2012; Clement et al., 2013; Aceti et al., 2015). We performed fast Fourier transform analysis to generate EEG power spectra of cortical brain waves in *Syngap1<sup>+/-</sup>* and WT mice during wake, NREM, and REM sleep. During wake, *Syngap1<sup>+/-</sup>* mice exhibited attenuated power in the alpha (8-12 Hz) and beta (12-20 Hz) frequency bands (main effect of genotype,  $F(1,23) = 20.007$ ,  $p = 0.0002$ ; Fig. 3.4a,b). In NREM sleep, *Syngap1<sup>+/-</sup>* mice display significantly elevated delta power (main effect of genotype:  $F(1,23) = 5.338$ ,  $p = 0.030$ ; Fig. 3.4c, genotype\*frequency interaction:  $F(77,1771) = 8.598$ ,  $p = 0.0002$ ; Fig. 3.4c), particularly in the low delta frequency band (0.5-1.5 Hz) (Mann-Whitney  $U = 5$ ,  $p = 0.00008$ ; Fig. 3.4d) compared to WT littermates. In REM sleep, *Syngap1<sup>+/-</sup>* mice have significantly reduced beta power (main effect of genotype,  $F(1,23) = 5.946$ ,  $p = 0.023$ ; Fig. 3.4e); however, there are no differences in theta power (4-8 Hz)—the most prominent wave form in REM sleep—between *Syngap1<sup>+/-</sup>* and WT mice ( $p = 0.83$ ; Fig. 3.4f). Despite the epileptic seizures observed in SYNGAP1 patients and the decreased seizure threshold of *Syngap1<sup>+/-</sup>* mice (Ozkan et al., 2014), we did not observe seizures in any of the mice during sleep or sleep deprivation.

***Syngap1<sup>+/-</sup> mice have less fluctuation of SWA activity across baseline, and have blunted homeostatic response following six hours of sleep deprivation***

Elevated NREM delta power in *Syngap1<sup>+/-</sup>* mice suggests that they may have abnormal homeostatic slow wave activity (SWA), an indicator of sleep depth that increases as a function of prior wakefulness (Borbély et al., 1981; Dijk et al., 1991; Achermann et



al., 1993; Dijk et al., 1993; Campbell et al., 2006). Indeed, across the 24-hour baseline, *Syngap1*<sup>+/-</sup> mice have less variance of delta power (0.5-4.0 Hz) (genotype\*time interaction,  $F(11,253) = 2.684$ ,  $p = 0.022$ ; Fig. 3.5a) and low delta power (0.5-1.5 Hz) (main effect of genotype,  $F(1,23) = 5.089$ ,  $p = 0.034$ ; Fig. 3.5b) across the day than WT mice. Following six hours of sleep deprivation by gentle handling, compared to WT mice, *Syngap1*<sup>+/-</sup> mice had significantly blunted homeostatic increase of delta (main effect of genotype,  $F(1,22) = 13.336$ ,  $p = 0.001$ ; Fig. 3.5c) and low delta SWA (main effect of genotype,  $F(1,22) = 6.599$ ,  $p = 0.018$ ; Fig. 3.5d), relative to baseline values. Blunted homeostatic SWA rebound in *Syngap1*<sup>+/-</sup> mice was contrasted by a larger increase in sleep time in *Syngap1*<sup>+/-</sup> mice during the first six hours following sleep deprivation, relative to baseline, compared to WT mice (genotype\*time interaction,  $F(2,52) = 3.466$ ,  $p = 0.039$ ; Fig. 3.5e). In the 18 hours following the culmination of sleep deprivation, there were no recovery sleep time differences, relative to baseline, between *Syngap1*<sup>+/-</sup> mice and WT controls (Student's t-test,  $p = 0.24$ ; Fig. 3.5f). Together, these data indicate that *Syngap1*<sup>+/-</sup> mice have abnormal homeostatic rebound following a sleep deprivation challenge.

### ***Syngap1*<sup>+/-</sup> mice exhibit slight home-cage hyperactivity and normal circadian periodicity**

Following recovery from sleep deprivation, all mice were transferred into individual home-cage beam break activity monitoring chambers. After one week of acclimation to the activity chambers, home-cage activity counts were binned into one-hour bins across the diurnal cycle and averaged over the week of recording. Relative to WT mice, *Syngap1*<sup>+/-</sup> mice were slightly but significantly hyperactive over the 24-hour day in both the horizontal (main effect of genotype,  $F(1,33) = 4.274$ ,  $p = 0.047$ ; Supplementary Fig. 3.2a)

and vertical direction (main effect of genotype,  $F(1,33) = 4.190$ ,  $p = 0.049$ ; Supplementary Fig. 3.2c). Analysis of activity counts separately in the light and dark parts of the cycle revealed no statistically significant activity differences between *Syngap1*<sup>+/-</sup> mice and WT littermates in either the horizontal ( $p = 0.10$ ; Supplementary Fig. 3.2b) or vertical axis ( $p = 0.17$ ; Supplementary Fig. 3.2d). After the last day of activity monitoring, one cohort of mice ( $n = 8$  WT and  $n = 7$  *Syngap1*<sup>+/-</sup>) remained in the activity monitoring chambers for 2 weeks of continuous darkness in order to access intrinsic circadian periodicity. There were no circadian period ( $\tau$ ) differences between *Syngap1*<sup>+/-</sup> and WT mice ( $p = 0.85$ ).

## Discussion

In this study, we investigated baseline sleep/wake, homeostatic recovery from sleep deprivation, and home-cage activity in male *Syngap1*<sup>+/-</sup> mice and WT littermates. Relative to WT, *Syngap1*<sup>+/-</sup> mice exhibited reductions in REM sleep time, quantities of NREM and REM bouts, and NREM to REM sleep transitions. EEG power spectra analysis revealed heightened delta power in NREM sleep and attenuated alpha and beta power during wake in comparison to WT mice. Following six hours of sleep deprivation, *Syngap1*<sup>+/-</sup> mice had less homeostatic rebound as measured by the change in NREM slow wave activity, relative to baseline. Finally, home-cage activity monitoring revealed slight home-cage hyperactivity in *Syngap1*<sup>+/-</sup> mice, but no circadian periodicity differences between *Syngap1*<sup>+/-</sup> mice and WT.

Reduced REM sleep total time and REM sleep bouts in *Syngap1*<sup>+/-</sup> mice is interesting for several reasons. REM sleep time reduction is one of the most commonly reported polysomnography findings in human ASD patients (Limoges et al., 2005; Buckley

et al., 2010), suggesting that the *Syngap1*<sup>+/-</sup> mouse model captures aspects of human ASD. Biochemical and slice physiology work in the mouse hippocampus suggests that REM sleep may be important for the consolidation of memories (Abel et al., 2013). *Syngap1*<sup>+/-</sup> mice have impaired hippocampal long-term potentiation (LTP), the presumptive cellular correlate of learning, following theta burst stimulation (Kim et al., 2003), which occurs physiologically during REM sleep. A recent study abrogating medial septal theta rhythms specifically during REM sleep impaired the consolidation of contextual fear memory in mice (Boyce et al., 2016). Interestingly, *Syngap1*<sup>+/-</sup> mice have normal 24-hour contextual fear memory, but have impaired remote contextual fear memory one week and one month after training (Gavin Rumbaugh, personal communications). These findings may be explained in part by the REM sleep reductions observed in *Syngap1*<sup>+/-</sup> mice in the present study, which may impair systems memory consolidation—a purported function of sleep.

EEG spectra alterations in *Syngap1*<sup>+/-</sup> mice are also fascinating for several reasons. Beta power is thought to be related to cognition, as beta power is increased in humans during sensory perception, motor planning, and mentally engaging tasks requiring attention and concentration (Sherman et al., 2016). SYNGAP1 mutant humans and *Syngap1*<sup>+/-</sup> mice exhibit cognitive deficits. Perhaps more interesting is the elevated delta power in *Syngap1*<sup>+/-</sup> mice, particularly in the low delta (0.5-1.5 Hz) frequencies. Delta rhythms are generated by the reciprocal interaction of thalamic reticular nucleus T-Type Ca<sup>2+</sup> channels and Ca<sup>2+</sup>-dependent small-conductance (SK)-type K<sup>+</sup> channels (Cueni et al., 2008; Lee et al., 2004). However, a second delta rhythm (<1.0 Hz), persists in the cortex of cats after thalamectomy, in cortical islands *in vivo*, and in cortical slices *in vitro*

(Steriade et al., 1993; Sanchez-Vives & McCormick, 2000; Timofeev et al., 2000). Slow wave delta activity is a measure of homeostatic drive and increases as a function of prior wakefulness (Borbély et al., 1981; Dijk et al., 1991; Achermann et al., 1993; Dijk et al., 1993; Campbell et al., 2006). Heightened delta power in baseline may suggest a “sleep deprived” brain in *Syngap1*<sup>+/-</sup> mice, and blunted homeostatic rebound following sleep deprivation may indicate difficulties responding to a sleep challenge. Chronically “sleep deprived” brain states in *Syngap1*<sup>+/-</sup> mice may model the default state observed in neurodevelopmental disorders, where insomnia and chronic insufficient sleep are frequent. Finally, elevated delta power in the basal state may be responsible for the REM sleep decrements observed in *Syngap1*<sup>+/-</sup> mice. The transition period from NREM to REM is marked by an increase in brain wave frequency (Saper et al., 2010) and transitions from deep NREM sleep (stage 3) to REM sleep are virtually non-existent in humans (Kishi et al., 2008). Thus, increased NREM delta power in *Syngap1*<sup>+/-</sup>, which is indicative of deeper sleep, may be inhibiting the ability to transition from NREM to REM sleep.

The diversity of sleep phenotypes in *Syngap1*<sup>+/-</sup> mice allows for promising future directions using spatially- and temporally- restricted SYNGAP1 hemideletion. REM sleep is thought to originate in brainstem nuclei, particularly the sublaterodorsal nucleus, ventrolateral periaqueductal gray, lateral pontine tegmentum, and ventral medulla (Sastre et al., 1996; Boissard et al., 2002; Lu et al., 2006; Luppi et al., 2006; Weber et al., 2015). Delta rhythms are generated in the thalamus (Cueni et al., 2008; Lee et al., 2004); however, a second delta rhythm in the lowest frequency bands (<1.0Hz), where the most significant delta power alterations in *Syngap1*<sup>+/-</sup> are observed, is generated locally in the cortex (Steriade et al., 1993). Thus, through conditional hemideletion of the *Syngap1*<sup>+/-</sup> protein using Cre-loxP technology, specific brain regions and circuits contributing to the

various sleep phenotypes outlined in this paper can be elucidated. Future sleep studies in conditional deletion mice, based on the findings from our present study, are likely to generate novel insight into the neurobiological mechanisms contributed to sleep problems reported in SYNGAP1 mutated humans, and possibly even to mechanisms underlying sleep disruptions in neurodevelopmental disorders more broadly.

**Author Contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Ted Abel and Gavin Rumbaugh. Christopher Angelakos, Ted Abel, and Gavin Rumbaugh designed the study. Christopher Angelakos did polysomnography surgeries. Christopher Angelakos and Jessica Schwarz conducted behavioral experiments and performed data analysis. Gavin Rumbaugh provided the mice used in this study.

## Figure Legends

### **Figure 3.1. *Syngap1*<sup>+/-</sup> mice have REM sleep reductions in comparison to wildtype (WT) mice.**

**A,B)** There are no differences in time spent awake between *Syngap1*<sup>+/-</sup> mice and WT. Expressed by light /dark cycle (A) and in 1-hour bins (B). **C,D)** *Syngap1*<sup>+/-</sup> mice have reduced NREM sleep relative to WT in the dark (active) phase. Expressed by light / dark cycle (C) and in 1-hour bins (D). **E,F)** *Syngap1*<sup>+/-</sup> mice have significantly reduced REM sleep relative to WT across the 24-hour day. Expressed by light /dark cycle (E) and in 1-hour bins (F). Mean  $\pm$  standard error of the mean (s.e.m.)

\*p < 0.05, \*\*p < 0.01

### **Figure 3.2. *Syngap1*<sup>+/-</sup> mice have decreased quantities of NREM and REM bouts compared to WT.**

**A)** There are no differences in the number of wake counts between *Syngap1*<sup>+/-</sup> and WT mice. **B)** There are no differences in the distribution of wake bout count duration between *Syngap1*<sup>+/-</sup> and WT. **C)** *Syngap1*<sup>+/-</sup> mice have significantly less NREM bouts than WT mice across the 24-hour day. **D)** *Syngap1*<sup>+/-</sup> mice have significantly less NREM bouts of the shortest duration (30s-80s and 80s-160s) compared to WT controls. **E)** *Syngap1*<sup>+/-</sup> mice have significantly reduced quantities of REM bouts relative to WT across the 24-hour day. **F)** *Syngap1*<sup>+/-</sup> mice have significantly fewer REM bouts of the shortest duration (20s-40s) in comparison to WT littermates. Mean  $\pm$  standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01

### **Figure 3.3. *Syngap1*<sup>+/-</sup> mice display less NREM to REM transitions than WT littermates.**

**A)** Across the diurnal cycle, *Syngap1*<sup>+/-</sup> mice exhibit fewer transitions from NREM to REM sleep than WT controls. **B)** There are no differences in the number of NREM to wake transitions between *Syngap1*<sup>+/-</sup> mice and WT littermates. **C)** There are no differences in the number of wake to NREM transitions between *Syngap1*<sup>+/-</sup> mice and WT. **D)** There are no differences in the number of REM

to Wake transitions between *Syngap1*<sup>+/-</sup> mice and WT. Mean ± standard error of the mean (s.e.m.)

\*p < 0.05, \*\*p < 0.01

**Figure 3.4. *Syngap1*<sup>+/-</sup> mice exhibit lower alpha and beta power during wake, and elevated delta power during NREM sleep than WT mice.** Left: 0.25 Hz bins from 0.5-20.0 Hz. Right: Binned into low delta (0.5-1.5Hz), delta (1.5-4.0Hz), theta (4.0-8.0Hz), alpha (8.0-12.0Hz), beta (12.0-20.0Hz), and gamma frequency bands (20.0-100.0Hz). **A,B)** *Syngap1*<sup>+/-</sup> mice have significantly reduced alpha and beta power during wake in comparison to WT mice. **C,D)** *Syngap1*<sup>+/-</sup> mice have significantly increased delta power during NREM sleep relative to WT controls. **E,F)** There are no significant differences in REM power spectra between *Syngap1*<sup>+/-</sup> mice and WT littermates, apart from a light reduction in beta power than it also observed during Wake and NREM sleep. Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

**Figure 3.5. *Syngap1*<sup>+/-</sup> mice have altered homeostatic sleep drive during both baseline sleep and following 6 hours of sleep deprivation.** **A,B)** *Syngap1*<sup>+/-</sup> mice have less fluctuation of NREM delta power (0.5-4.0Hz) (A) and low delta power (0.5-1.5Hz) (B) across the 24-hour baseline diurnal cycle than WT mice. **C,D)** *Syngap1*<sup>+/-</sup> mice have significantly less slow wave activity (C: delta power; D: low delta power) rebound following 6 hours of sleep deprivation than WT mice. **E)** Compared to WT, *Syngap1*<sup>+/-</sup> mice exhibit a significantly lower increase of NREM sleep time, relative to baseline, during the first 6 hours of recovery following sleep deprivation. **F)** There are no differences in recovery NREM sleep time, relative to baseline, between WT and *Syngap1*<sup>+/-</sup> over the course of the 18 hours following sleep deprivation. Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001



## Supplementary Figure Legends

**Supplemental Figure 3.1. *Syngap1*<sup>+/-</sup> mice have normal mean durations of Wake, NREM, and REM sleep bouts.** **A,B)** There are no differences in mean wake bout duration (A) or temporal distribution of wake bouts (B) between *Syngap1*<sup>+/-</sup> and WT mice. **C,D)** There are no differences in mean NREM bout duration (C) or temporal distribution of NREM bouts (D) between *Syngap1*<sup>+/-</sup> and WT mice. **E)** There are no differences in mean REM bout duration between SYGNAP1<sup>+/-</sup> and WT mice. **F)** Relative to WT mice, SYGAP1<sup>+/-</sup> mice have a slightly altered temporal distribution of REM bouts, spending a lower % of REM sleep time in bouts of the shortest duration (20s-40s) and a slightly higher proportion of REM sleep time in bouts 40s-60s in duration. Mean ± standard error of the mean (s.e.m.) \*p < 0.05.

**Supplemental Figure 3.2. *Syngap1*<sup>+/-</sup> are hyperactive in the home-cage in comparison to WT mice.** **A,B)** SYNGAP2<sup>+/-</sup> exhibit home-cage ambulatory hyperactivity in the XY (horizontal) axis, relative to WT. Expressed in 1-hour bins (A) and binned by light / dark cycle (B). **C,D)** *Syngap1*<sup>+/-</sup> mice exhibit significantly more rearing (vertical) activity than WT mice. Expressed in 1-hour bins (C) and binned by light / dark cycle (D). Mean ± standard error of the mean (s.e.m.) \*p < 0.05.

**Supplemental Figure 3.3. Normal circadian rhythms in *Syngap1*<sup>+/-</sup> mice.** **A)** There are no differences in free-running circadian period (tau) between SYGNAP1<sup>+/-</sup> mice and WT littermates. **B,C)** Representative actograms across the two weeks of constant darkness for WT mice (B) and *Syngap1*<sup>+/-</sup> mice (C). Mean ± standard error of the mean (s.e.m.)

**Figure 3.1**

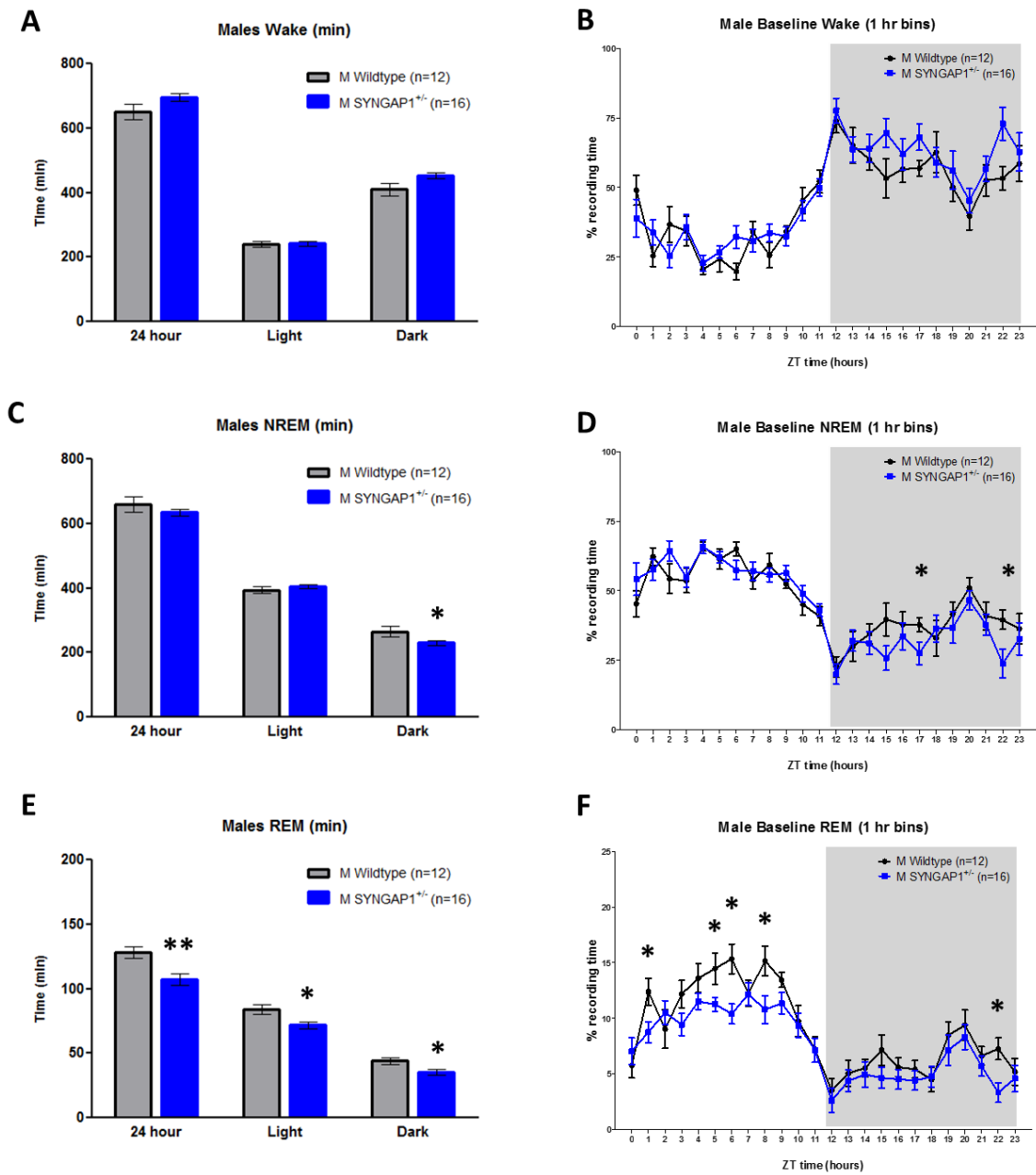
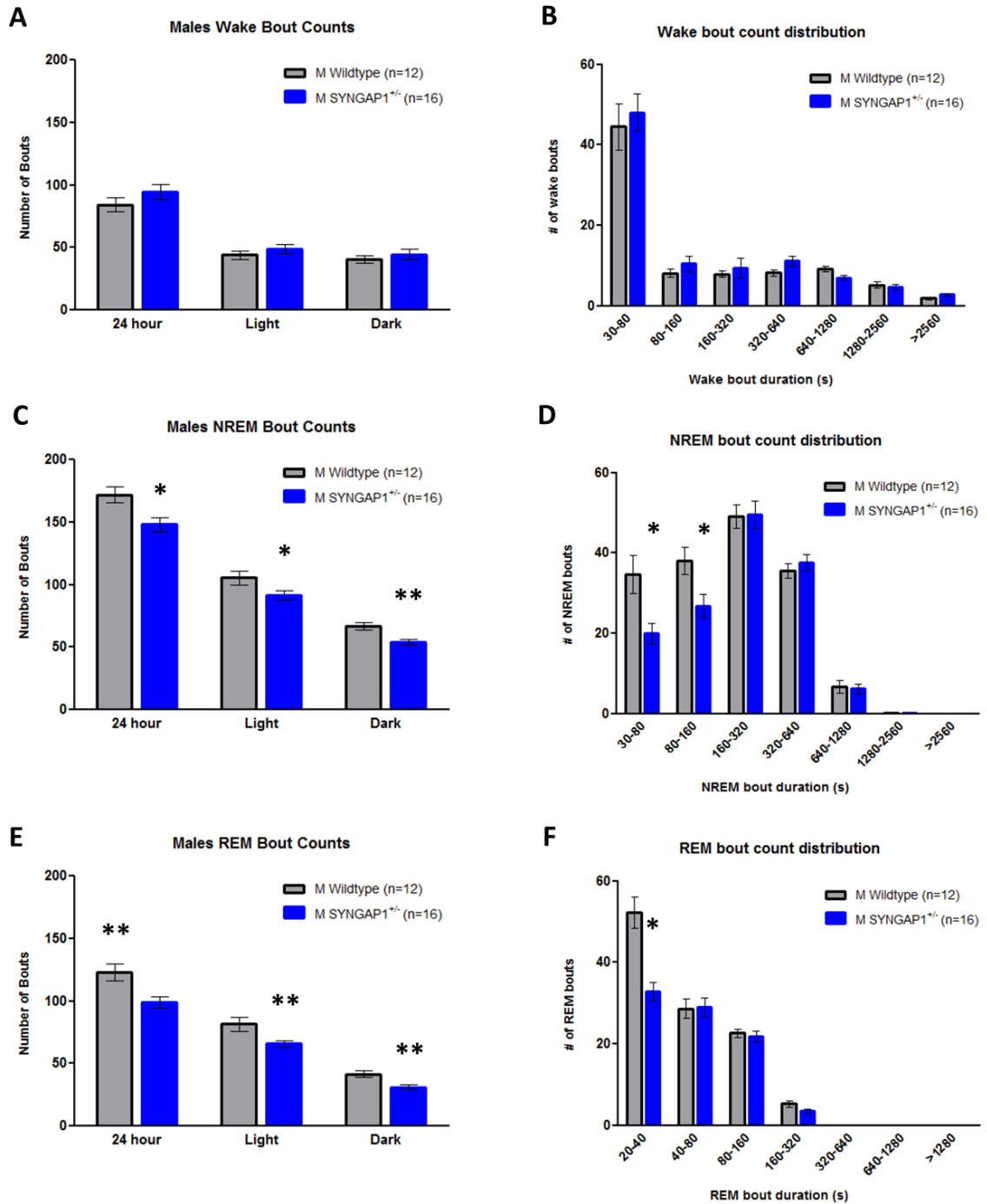
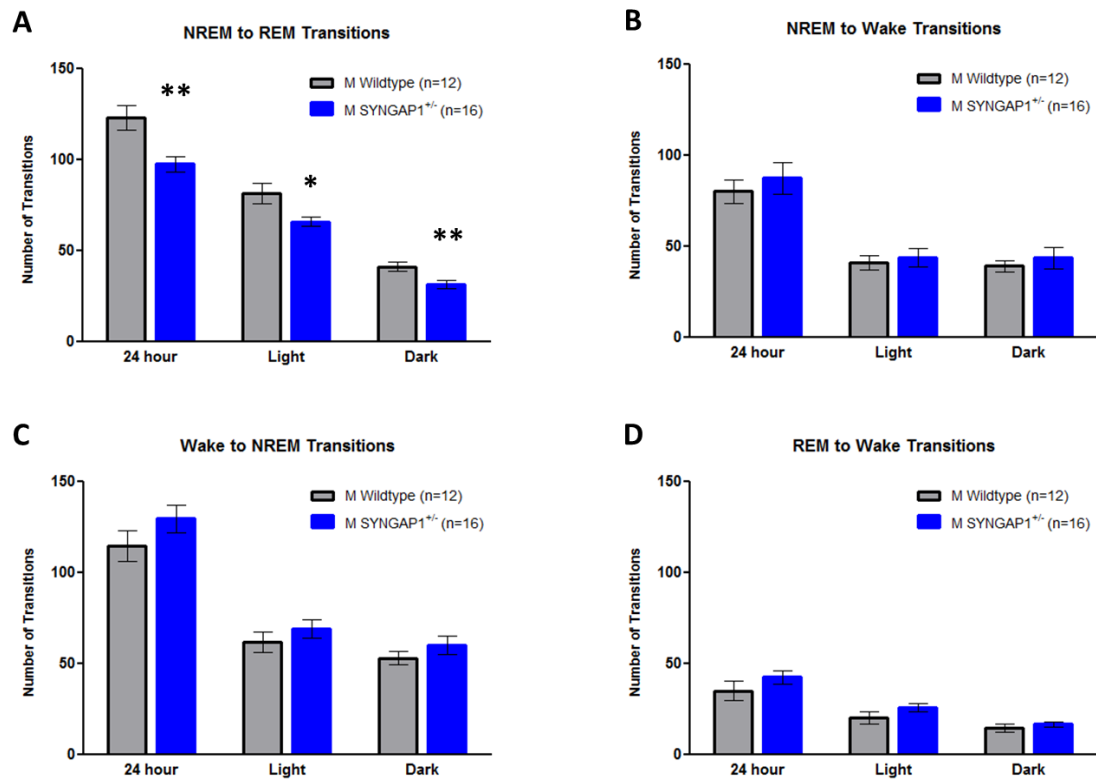


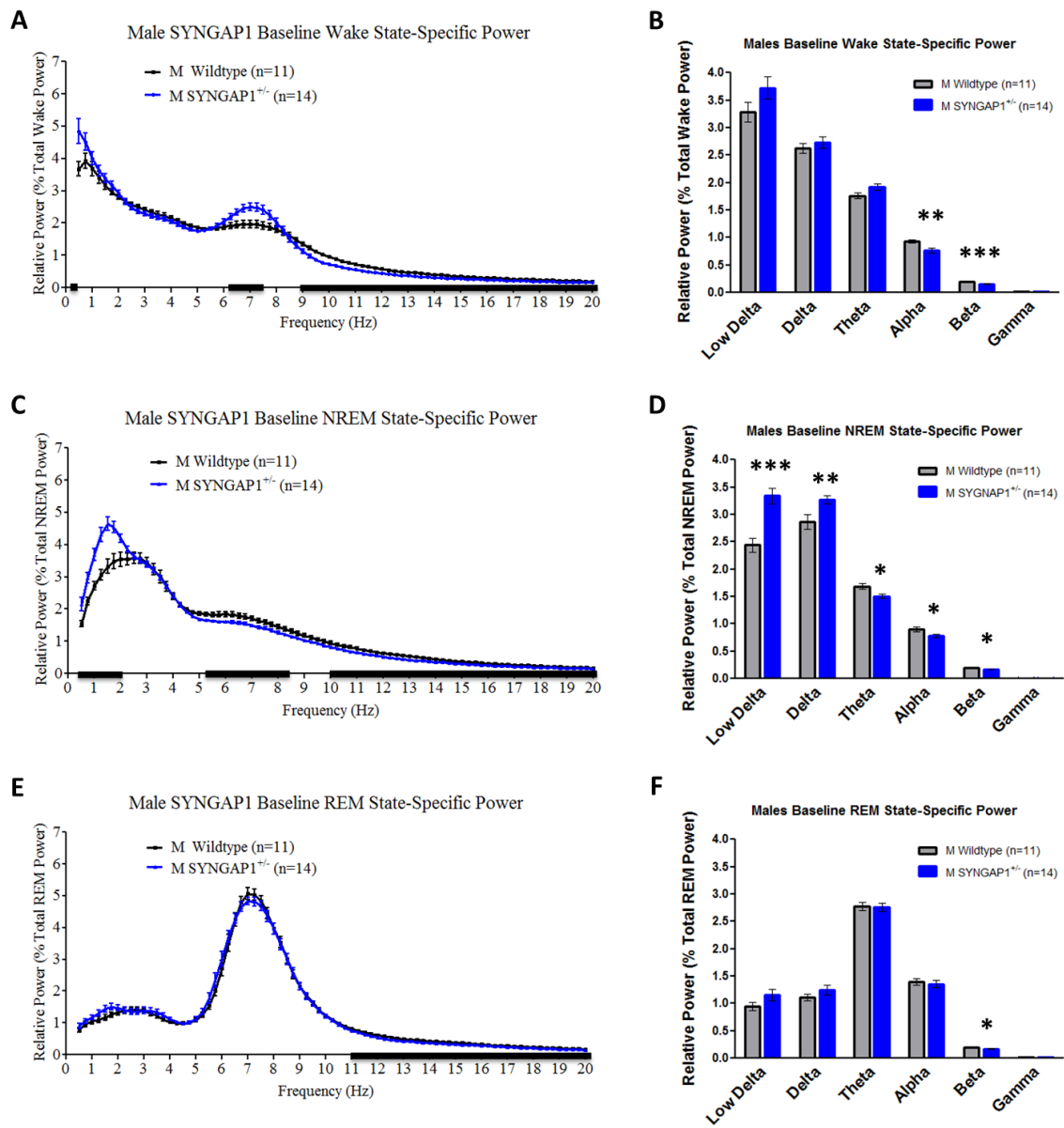
Figure 3.2



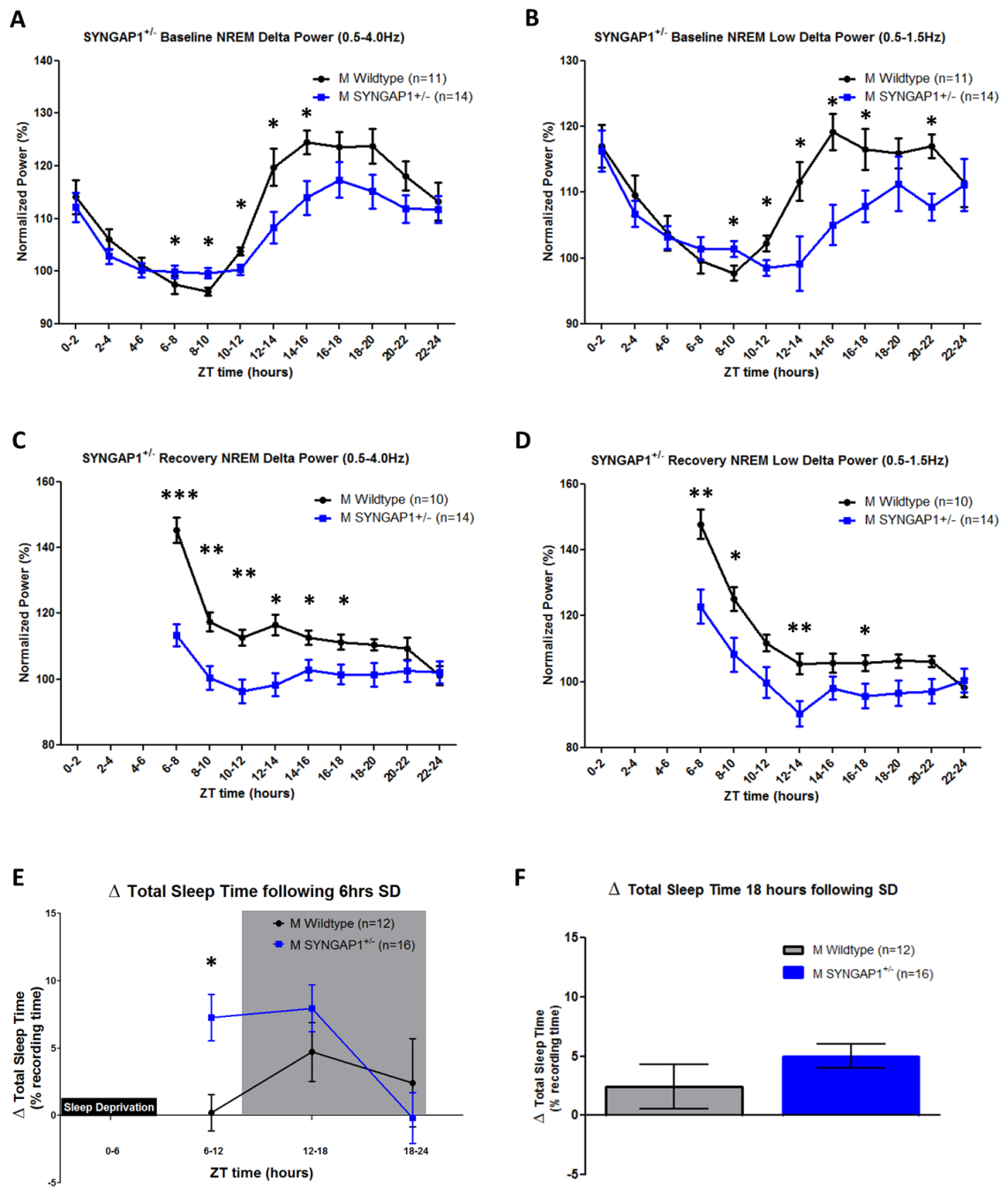
**Figure 3.3**



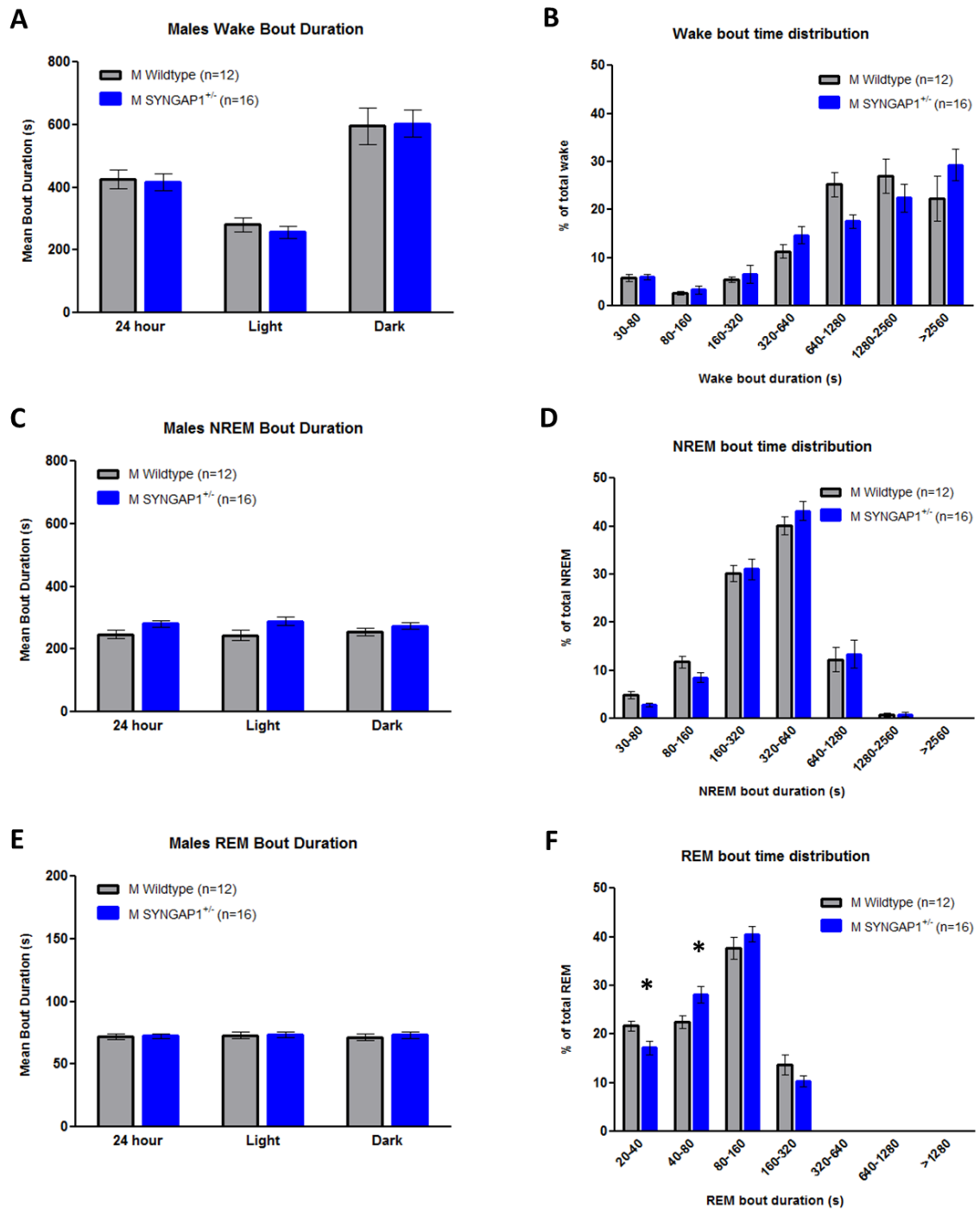
**Figure 3.4**



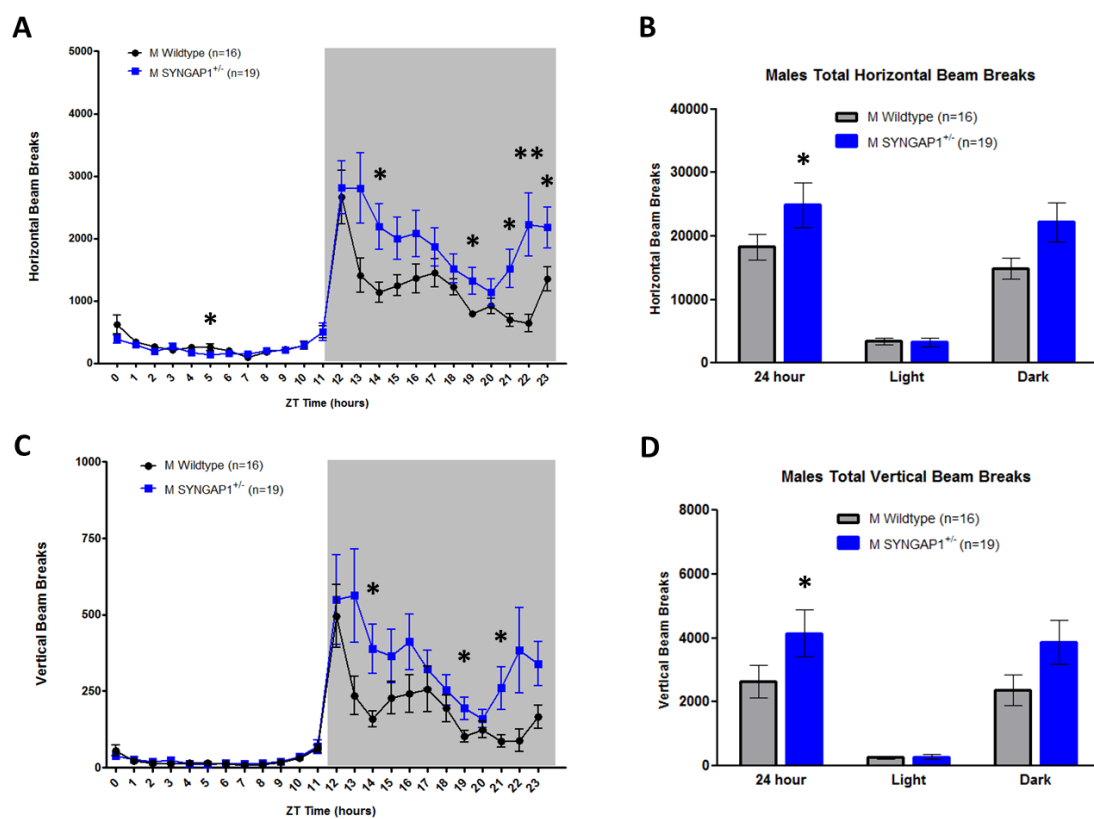
**Figure 3.5**



Supplemental Figure 3.1

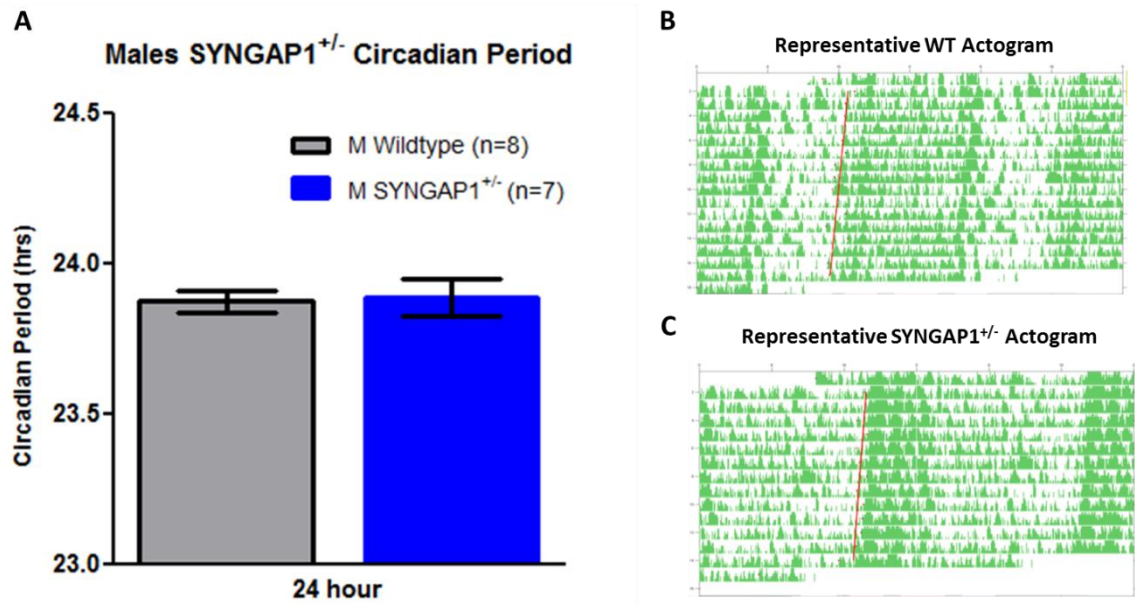


Supplemental Figure 3.2





Supplemental Figure 3.3



## CHAPTER 4: Home-cage hypoactivity in four mouse models of autism spectrum disorder

### Abstract

Genome-wide association studies from Autism Spectrum Disorder (ASD) patient populations have implicated numerous risk factor genes whose mutation or deletion results in significantly increased prevalence of ASD. Behavioral studies of monogenic mutant mouse models of ASD-associated genes have been useful for identifying aberrant neural circuitry. However, behavioral results often differ from lab to lab, and direct comparisons between males and females are often not performed despite the significant sex-bias of ASD. In this study, we sought to investigate the simple, passive behavior of home-cage activity monitoring across multiple 24-hour days in four different monogenic mouse models of ASD: *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO mice. Relative to sex-matched WT littermates, we discovered significant home-cage hypoactivity, particularly in the dark (active) phase of the light/dark cycle in male mice of all four ASD-associated transgenic models. For *CNTNAP2*<sup>-/-</sup> and *Pcdh10*<sup>+/-</sup> mice, these activity alterations were sex-specific, as female mice did not exhibit home-cage activity differences relative to sex-matched WT controls. These home-cage hypoactivity alterations differ from, and in some cases, contradict activity findings previously reported using short-term activity measurements in a novel arena. Despite circadian problems reported in human ASD patients, none of the mouse models studied had alterations in free-running circadian period. Together, these findings highlight a shared phenotype across several monogenic mouse models of ASD, outline the importance of methodology

on behavioral interpretation, and in some cases, parallel the male-enhanced phenotypic presentation found in human ASDs.

## Introduction

Autism spectrum disorder (ASD) has an estimated prevalence of 1 in 68 births and is substantially sex-biased, with males 4.5 times more likely than females to receive an ASD diagnosis (Christensen et al., 2016). Mouse genetic models have been an invaluable tool for studying behavioral, electrophysiological, and molecular mechanisms contributing to ASD (see Bey & Jiang, 2014; Ellegood & Crawley, 2015; Hulbert & Jiang, 2016 for recent reviews). Behavioral phenotypes in rodent models of neurodevelopmental disorders help pinpoint potential brain regions and circuitry underlying symptomology. Unfortunately, behavioral methodologies and results within the same genetic model often differ from lab to lab, confounding interpretation (Wahlsten et al., 2003).

In this study, we quantified home-cage activity and circadian rhythms across multiple 24-hour days in males and females of four transgenic mouse models of ASD: *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO. SH3 and multiple Ankyrin repeat domains 3 (Shank3) is a scaffolding protein in the postsynaptic density whose deletion contributes to Phelan-McDermind syndrome, intellectual disability, and ASD-like behaviors in humans (Durand et al., 2007) and social deficits and repetitive behaviors in mice (Peça et al., 2011a). Contactin associated protein-like 2 (CNTNAP2) is a member of the neurexin superfamily and is associated with potassium channels, myelination, and neuron-glia interaction (Poliak et al., 1999). Its deletion results in cortical dysplasia-focal epilepsy, seizures, and ASD in humans (Strauss et al., 2006), and seizures, social deficits, motor stereotypies, communication abnormalities, and interneuron reductions in mice (Peñagarikano et al., 2011). Procadherin-10 is a member of the cadherin superfamily of cell-adhesion molecules whose reduction has been associated with ASD in human

genome-wide association studies (Morrow et al., 2008; Bucan et al., 2009), and altered communication, male-specific social deficits, and dendritic spine morphology alterations of the lateral / basolateral amygdala in *Pcdh10<sup>+/-</sup>* mice (Schoch et al., 2017a). *Fmr1* encodes for the Fragile X mental retardation protein and is one of the leading single-gene causes of autism and intellectual disability (Turner et al., 1996; Hagerman et al., 2008). Fragile X patients display intellectual impairment, social deficits, communication problems, anxiety, hyperarousal, and hyperactivity (Garber et al., 2008; Yu & Berry-Kravis, 2014) and *Fmr1* KO mice have learning deficits, abnormal social behavior, anxiety, altered dendritic spine development, and aberrant cortical synchrony (Bakker et al., 1994; Comery et al., 1997; Spencer et al., 2005; Gonçalves et al. 2013).

The goals of this home-cage activity monitoring study were three-fold: 1) To investigate the same behavior in multiple ASD models in the same lab environment, using consistent methodologies. 2) To examine both males and females given the pronounced sex-bias in ASD diagnoses (Christensen et al., 2016). 3) To compare findings with published activity results— specifically comparing and contrasting continuous home-cage activity across multiple diurnal days with brief activity monitoring in a novel environment.

## **Materials and Methods**

### *Animals*

*Shank3B<sup>-/-</sup>* mice were generated as previously described (Peça et al., 2011). Briefly, exons 13-16 of *Shank3* were replaced with a neo cassette. B6.129-*Shank3<sup>tm2Gfng</sup>/J* (Stock #017688) heterozygous males and females were purchased from The Jackson Laboratory and mated together to generate *Shank3B<sup>-/-</sup>* offspring and WT controls. *Pcdh10<sup>+/-</sup>* mice

were generated as previously described (Uemura et al., 2007; Schoch et al., 2017). *Pcdh10*<sup>+/-</sup> mice in which the first exon of *Pcdh10* was replaced with a lacZ-neo cassette were created by Lexicon Pharmaceuticals, Inc. (Basking Ridge, NJ). Founders were backcrossed for more than 15 generations before male *Pcdh10*<sup>+/-</sup> mice were mated with C57Bl/6J female mice purchased from the Jackson Laboratory (Stock #000664) to produce heterozygous *Pcdh10*<sup>+/-</sup> experimental animals and *Pcdh10*<sup>+/+</sup> wildtype controls. *Fmr1* KO mice were generated as previously described (Bakker et al., 1994). Briefly, exon 5 of *Fmr1* was replaced with a neo cassette and resulting males were backcrossed with C57Bl/6J females for many generations. B6.129P2-*Fmr1*<sup>tm1Cgr</sup>/J female mice (Stock #003025) were purchased from The Jackson Laboratory and mated with C57Bl/6J (stock #000664) males. Because Fragile X is an X-linked condition, and females display much milder and more variable symptoms than males due to random X-inactivation, (Loesch et al., 2004; Yu & Berry-Kravis, 2014), only male *Fmr1* KO mice were studied. *CNTNAP2*<sup>-/-</sup> mice were generated as previously described (Poliak et al., 2003). Exon 1 of *Caspr2* was replaced with a neo cassette and resulting males were backcrossed to C57Bl/6J for 10-12 generations (Peñagarikano et al., 2011). Heterozygous B6.129(Cg)-*Cntnap2*<sup>tm1Pele</sup>/J males and females from The Jackson Laboratory (Stock #017482) were mated together to produce experimental *CNTNAP2*<sup>-/-</sup> males and females and WT controls. Mice for all lines were weaned at 21 days old in cages of 3-5 sex-matched littermates. All mice were between 2 and 4.5 months old at the beginning of activity monitoring experimentation. All animals were maintained on a 12 hour light: 12 hour dark cycle (lights on at 7:00am), except where indicated otherwise for circadian studies in constant darkness, with food and water provided *ad libitum*. All experiments were approved by the University of

Pennsylvania Institutional Care and Use Committee and conducted in accordance to National Institute of Health guidelines.

### *Activity Monitoring*

Activity monitoring was performed as previously described (Angelakos et al., 2016). Mice were single housed within individual noise- and light-attenuating chambers (22" x 16" x 19", Med Associates, St. Albans, VT) equipped with a 250 lux light source (80 lux at cage floor), ventilation fan, and an infrared beam break system which surrounded the mouse cage on all four sides and provided a high-resolution scaffold of infrared beams and detectors. Infrared beams were spaced 0.5" apart and provided two horizontal grids at 0.75" and 2.75" from the cage floor to quantify horizontal and vertical (rearing) activity, respectively. Mice were allowed to acclimate to the chambers for one week before experimental testing. Following acclimation, activity counts (beam breaks) were tabulated in 10 second intervals in both the XY (vertical) and Z (vertical) direction continuously for 7 days in 12 hour light: 12 hour dark (12h:12h LD). Activity counts were pooled into 1 hour bins across the entire diurnal cycle and averaged over the course of the 7 days. After 7 days of activity monitoring in 12h:12h LD, lights were switched off for 2 consecutive weeks of constant darkness (DD) to quantify free-running circadian period ( $\tau$ ). Circadian  $\tau$  was calculated from Day 2 to Day 14 of DD using Clocklab software (Actimetrics).

### *Statistics*

All statistical analysis was performed using SPSS for Windows (V. 24.0). To analyze home-cage activity (1-hour bins), Mixed Design ANOVAs were utilized with genotype as the between-subjects factor (WT or transgenic) and time as the within-

subjects factor. *Post hoc* multiple comparisons were performed using Bonferroni's adjustment for multiple comparisons. Where the assumption of sphericity was violated, Greenhouse-Geisser corrected F values are given. Multivariate ANOVAs (MANOVAs) were performed to analyze activity counts in the light cycle and dark cycle, with alphas corrected for multiple ANOVAs and set at  $\alpha = 0.05/2$ , followed by *post hoc* Bonferroni's adjustment for multiple comparisons. Student's T-test was used to compare Circadian Tau values between WT and transgenic mice.

## Results

### ***Home-cage hypoactivity in male Shank3B<sup>-/-</sup>, CNTNAP2<sup>-/-</sup>, Pcdh10<sup>+/-</sup>, and Fmr1 KO mice***

Home-cage beam break counts were tabulated over 7 days of continuous monitoring in 12h:12h LD in male experimental mice and sex-matched WT controls of the four transgenic models of autism spectrum disorder: Shank3B<sup>-/-</sup>, CNTNAP2<sup>-/-</sup>, Pcdh10<sup>+/-</sup>, and Fmr1 KO. In the horizontal (XY) direction, compared to WT, ASD-associated genetic deletion resulted in decreased home-cage activity for male Shank3B<sup>-/-</sup> (main effect of genotype;  $F(1,18) = 14.466$ ,  $p = 0.001$ , Fig. 4.1a), CNTNAP2<sup>-/-</sup> (time\*genotype interaction:  $F(23,467) = 4.409$ ,  $p = 0.003$ , Fig. 4.1c), Pcdh10<sup>+/-</sup> (main effect of genotype:  $F(1,20) = 10.562$ ,  $p = 0.004$ , Fig. 4.1e), and Fmr1 KO mice (main effect of genotype:  $F(1,25) = 8.125$ ,  $p = 0.009$ , Fig. 4.1g). Analysis by light / dark cycle revealed significantly reduced activity specifically in the dark (active) phase for Shank3B<sup>-/-</sup> (MANOVA,  $F(2,17) = 8.093$ ,  $p = 0.003$ , Fig. 4.1b), Pcdh10<sup>+/-</sup> (MANOVA,  $F(2,19) = 6.331$ ,  $p = 0.008$ , Fig. 4.1f), and Fmr1 KO males (MANOVA:  $F(2,24) = 26.801$ ,  $p < 0.000001$ , Fig. 4.1h), relative to WT littermates. The



magnitude of activity differences between male WT and ASD model littermates was even larger in the vertical (Z) direction for all lines studied (Supplementary Figure 4.1). Thus, reduction of four different ASD-associated genes resulted in decreased home-cage ambulatory and rearing activity in male mutant mice relative to sex-matched controls.

***Shank3B<sup>-/-</sup> female mice exhibit home-cage hypoactivity, but female CNTNAP2<sup>-/-</sup> and Pcdh10<sup>+/-</sup> have normal activity levels***

Because ASD is a male-biased disorder and females often exhibit less severe symptomology than males even under the same genetic insult (Robinson et al., 2013; Werling & Geschwind, 2013; Jacquemont et al., 2014), we also quantified home-cage activity behavior in female mice and sex-matched littermates to look for potential sex differences. Female *Fmr1* KO mice were not studied because Fragile X is an X-linked condition and phenotypes in females are less severe, more variable, and less predictable than males due to random X-inactivation (Loesch et al., 2004; Yu & Berry-Kravis, 2014). Similar to *Shank3B<sup>-/-</sup>* male mice, *Shank3B<sup>-/-</sup>* females exhibited robust home-cage hypoactivity relative to WT (main effect of genotype:  $F(1,18) = 20.447$ ,  $p < 0.001$ , Fig. 4.2a). Analysis by light / dark cycle indicated that *Shank3B<sup>-/-</sup>* females were less active than WT females in both the light and dark phases (MANOVA,  $F(2,17) = 9.699$ ,  $p = 0.002$ , dark phase: Fig. 4.2b). Interestingly, unlike male *CNTNAP2<sup>-/-</sup>* and *Pcdh10<sup>+/-</sup>* mice, there were no activity differences relative to sex-matched WT littermates in female *CNTNAP2<sup>-/-</sup>* mice ( $F(1,19) = 0.033$ ,  $p = 0.86$ , Fig. 4.2c,d) or *Pcdh10<sup>+/-</sup>* mice ( $F(1,20) = 1.960$ ,  $p = 0.18$ , Fig. 4.2e,f). These female activity findings were similar in rearing behavior (Supplementary Figure 4.2). Altogether, activity alterations were male-specific for two of the four ASD-

associated mouse models studied, consistent with heightened ASD symptomology in males compared to females with the same genetic insult.

### ***Circadian rhythms are unaltered in the four ASD mouse models***

Circadian issues are common in ASD patients, who often have difficulties falling asleep and have more frequent night awakenings than the control population (Richdale & Prior, 1995; Glickman, 2010). Because of this, we sought to investigate intrinsic circadian period of the ASD-associated transgenic mouse lines. Following two weeks of continuous activity monitoring, lights were shut off and mice were allowed to free-run in continuous darkness for two weeks. Despite activity alterations found in the ASD mouse models, there were no differences in free-running circadian period between male and female transgenic mice and sex-matched littermates for any of the four ASD-associated lines studied (Student's T-Test,  $p > 0.25$  for all comparisons, Supplementary Fig. 4.3).

### **Discussion**

In this study, we performed home-cage activity monitoring and circadian behavioral analysis in male and female mice of four transgenic mouse models of ASD. Surprisingly, male mice of all four lines studied—*Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO—exhibited home-cage hypoactivity, particularly in the dark (active) phase of the diurnal cycle, relative to sex-matched WT controls. Interestingly, for *CNTNAP2*<sup>-/-</sup> and *Pcdh10*<sup>+/-</sup> mice, the activity alterations were sex-specific, as female mice of these lines displayed activity levels similar to sex-matched WT littermates. The enhanced activity alterations in males for these lines parallels sex differences in phenotypic presentation in human ASD patients (Werling & Geschwind, 2013) and supports findings that females require a larger

mutational burden than males to manifest similar ASD-related symptomology (Robinson et al., 2013; Jacquemont et al., 2014). To our knowledge, this is the first study to include and directly compare male and female home-cage activity in these ASD mouse lines across the diurnal cycle.

Another major finding from this study is the importance of dissociating continuous, undisturbed home-cage activity from novelty-induced, brief activity measurements that may contain anxiogenic elements, such as those obtained in an open field test. *Fmr1* KO mice have been described as hyperactive in the open field in numerous publications (Bakker et al., 1994; Peier et al., 2000; Mineur et al., 2002; Restivo et al., 2005; Spencer et al., 2005; Dahlhaus & El-Husseini, 2010; Yuskaitis et al., 2010; Liu et al., 2011; Pietropaolo et al., 2011; Ding et al., 2014; Gholizadeh et al., 2014; Uutela et al., 2014). Likewise, *CNTNAP2*<sup>-/-</sup> mice have been reported as hyperactive in brief (<1 hour) activity monitoring sessions (Peñagarikano et al., 2011; Brunner et al., 2015). However, longer activity studies across multiple undisturbed days corroborate our findings of hypoactivity in *Fmr1* KO (Bonasera et al., 2017) and *CNTNAP2*<sup>-/-</sup> male mice (Thomas et al., 2016). *Shank3B*<sup>-/-</sup> mice have previously been reported to be hypoactive (Kouser et al., 2013; Lee et al., 2015; Bidinosti et al., 2016; Mei et al., 2016; Copping et al., 2017; Dhamne et al., 2017), however numerous other labs have reported no open field activity differences between *Shank3B*<sup>-/-</sup> males and WT controls (Peça et al., 2011; Drapeau et al., 2014; Duffney et al., 2015; Jaramillo et al., 2016). In some instances investigating both *Shank3B*<sup>-/-</sup> males and females in the open field, males were found to be hypoactive, but females were not (Wang et al., 2011; M. Yang et al., 2012). Finally, activity has not previously been studied in *Pcdh10*<sup>+/-</sup> mice except during social behavior (Schoch et al., 2017), but we have

not observed activity differences in the open field (data not shown). Clearly, these results highlight the differences between undisturbed home-cage activity over multiple days and brief activity measures in a novel environment, and caution against the use and reliability of open field to determine whether an animal has altered basal activity.

Our findings also present a shared phenotype across multiple mouse models of ASD, performed in one lab using consistent methodologies. While human Fragile X patients and those with CNTNAP2 mutation are hyperactive (Baumgardner et al., 1995; Strauss et al., 2006; Sullivan et al., 2006; Tranfaglia, 2011), the consistent phenotype of activity alterations in ASD patients and mouse models may suggest aberrant corticostriatal connectivity. Corticostriatal circuits play a role in coordinated movement, and alterations in this circuitry are believed to be one of major structural regions contributing to attention-deficit hyperactivity disorder (Cubillo et al., 2012; Curatolo et al., 2010; Nakao et al., 2011) and ASD (Langen et al., 2012; Shepherd, 2013), which are highly comorbid (Antshel et al., 2013). One limitation of our study is that all activity and circadian measurements were performed in adult mice, and ASD is a neurodevelopmental disorder. Future studies investigating activity across development may shed light into the precise circuitry and mechanisms underlying activity alterations, and perhaps other behavioral deficits common between Shank3B, CNTNAP2<sup>+/-</sup>, Pcdh10<sup>+/-</sup>, and Fragile X mice and humans.

**Author contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Jennifer Tudor, Sarah Ferri, and Ted Abel. Christopher Angelakos and Ted Abel designed the study. Christopher Angelakos, Jennifer Tudor, and Sarah Ferri conducted behavioral experiments. Christopher Angelakos analyzed activity and circadian behavioral data.

## Figure Legends

**Figure 4.1. Male *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO mice are hypoactive in the home-cage relative to sex-matched WT controls. A,B)** *Shank3B*<sup>-/-</sup> males exhibit significantly reduced horizontal activity compared to WT littermates. Expressed in 1 hour bins (A) and by light / dark cycle (B). **C,D)** *CNTNAP2*<sup>-/-</sup> males have decreased horizontal activity compared to WT controls. Expressed in 1 hour bins (C) and by light / dark cycle (D). **E,F)** *Pcdh10*<sup>+/-</sup> males display significantly less horizontal activity than WT littermates. Expressed in 1 hour bins (E) and by light / dark cycle (F). **G,H)** *Fmr1* KO males demonstrate significantly lower horizontal activity than WT controls. Expressed in 1 hour bins (G) and by light / dark cycle (H). Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

**Figure 4.2. Female *Shank3B*<sup>-/-</sup> are hypoactive, but female *CNTNAP2*<sup>+/-</sup> and *Pcdh10*<sup>+/-</sup> display no home-cage activity differences, relative to sex-matched WT controls. A,B)** *Shank3B*<sup>-/-</sup> females exhibit significantly reduced horizontal activity compared to WT littermates. Expressed in 1 hour bins (A) and by light / dark cycle (B). **C,D)** *CNTNAP2*<sup>+/-</sup> females have no differences in horizontal activity compared to WT controls. Expressed in 1 hour bins (C) and by light / dark cycle (D). **E,F)** *Pcdh10*<sup>+/-</sup> females display no differences in horizontal activity in comparison to WT littermates. Expressed in 1 hour bins (E) and by light / dark cycle (F). Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

## Supplementary Figure Legends

**Supplemental Figure 4.1. Male *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO mice display less home-cage rearing behavior than sex-matched WT controls. A,B) *Shank3B*<sup>-/-</sup> males exhibit significantly reduced vertical activity compared to WT littermates. Expressed in 1 hour bins (A) and by light / dark cycle (B). C,D) *CNTNAP2*<sup>-/-</sup> males have decreased vertical activity compared to WT controls. Expressed in 1 hour bins (C) and by light / dark cycle (D). E,F) *Pcdh10*<sup>+/-</sup> males display significantly less vertical activity than WT littermates. Expressed in 1 hour bins (E) and by light / dark cycle (F). G,H) *Fmr1* KO males demonstrate significantly lower vertical activity than WT controls. Expressed in 1 hour bins (G) and by light / dark cycle (H). Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001**

**Supplemental Figure 4.2. Female *Shank3B*<sup>-/-</sup> have reduced rearing behavior, but female *CNTNAP2*<sup>+/-</sup> and *Pcdh10*<sup>+/-</sup> display no vertical activity differences, relative to sex-matched WT controls. A,B) *Shank3B*<sup>-/-</sup> females exhibit significantly reduced vertical activity compared to WT littermates. Expressed in 1 hour bins (A) and by light / dark cycle (B). C,D) *CNTNAP2*<sup>+/-</sup> females have no differences in vertical activity compared to WT controls. Expressed in 1 hour bins (C) and by light / dark cycle (D). E,F) *Pcdh10*<sup>+/-</sup> females display no differences in vertical activity in comparison to WT littermates. Expressed in 1 hour bins (E) and by light / dark cycle (F). Mean ± standard error of the mean (s.e.m.) \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001**

**Supplemental Figure 4.3. Normal circadian rhythms in *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and *Fmr1* KO mice. A-D) There are no differences in free-running circadian period (tau) between *Shank3B*<sup>-/-</sup> (A), *CNTNAP2*<sup>-/-</sup> (B), *Pcdh10*<sup>+/-</sup> (C), *Fmr1* KO (D) mice and their sex-matched WT controls.**

Figure 4.1

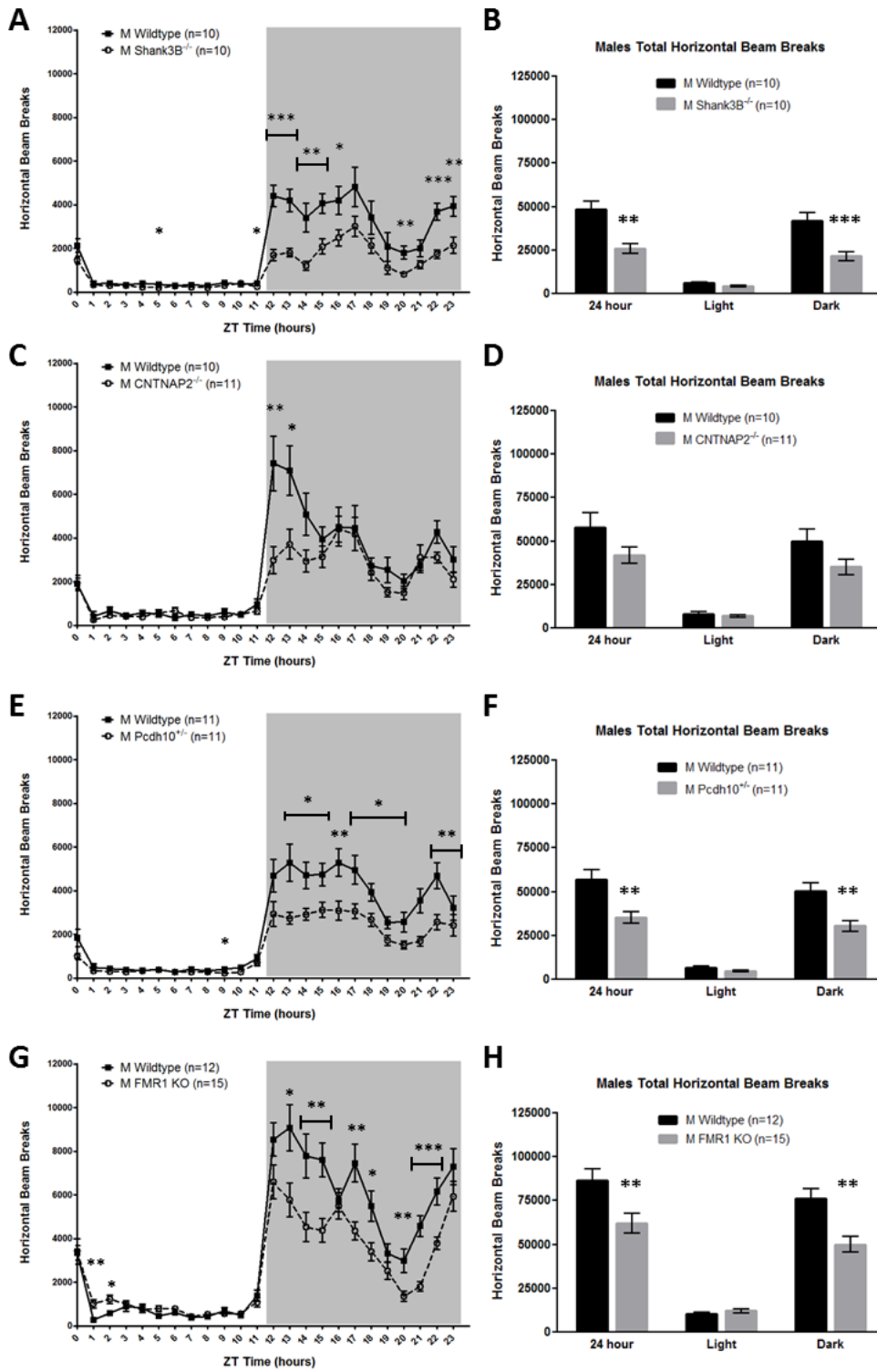
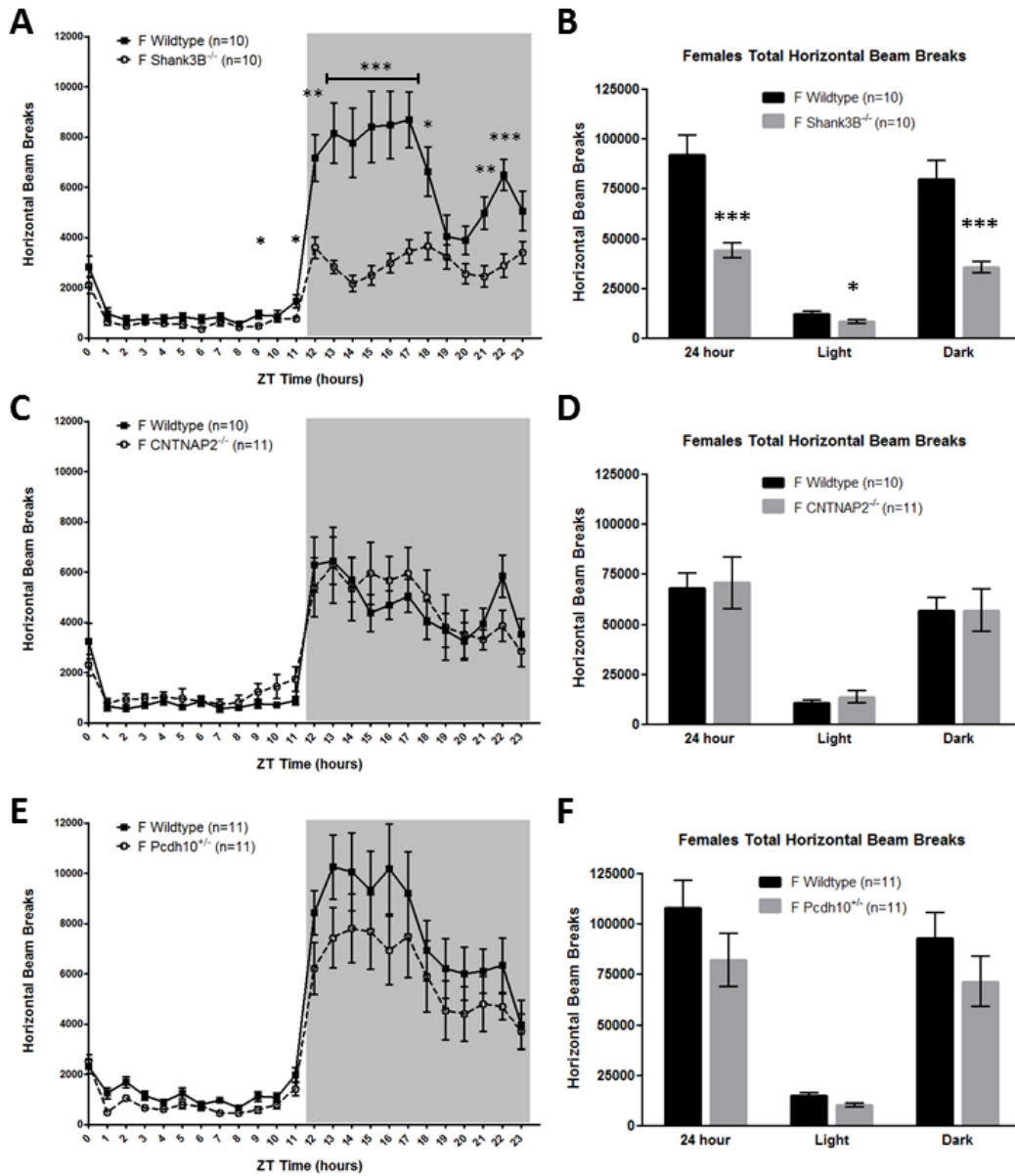
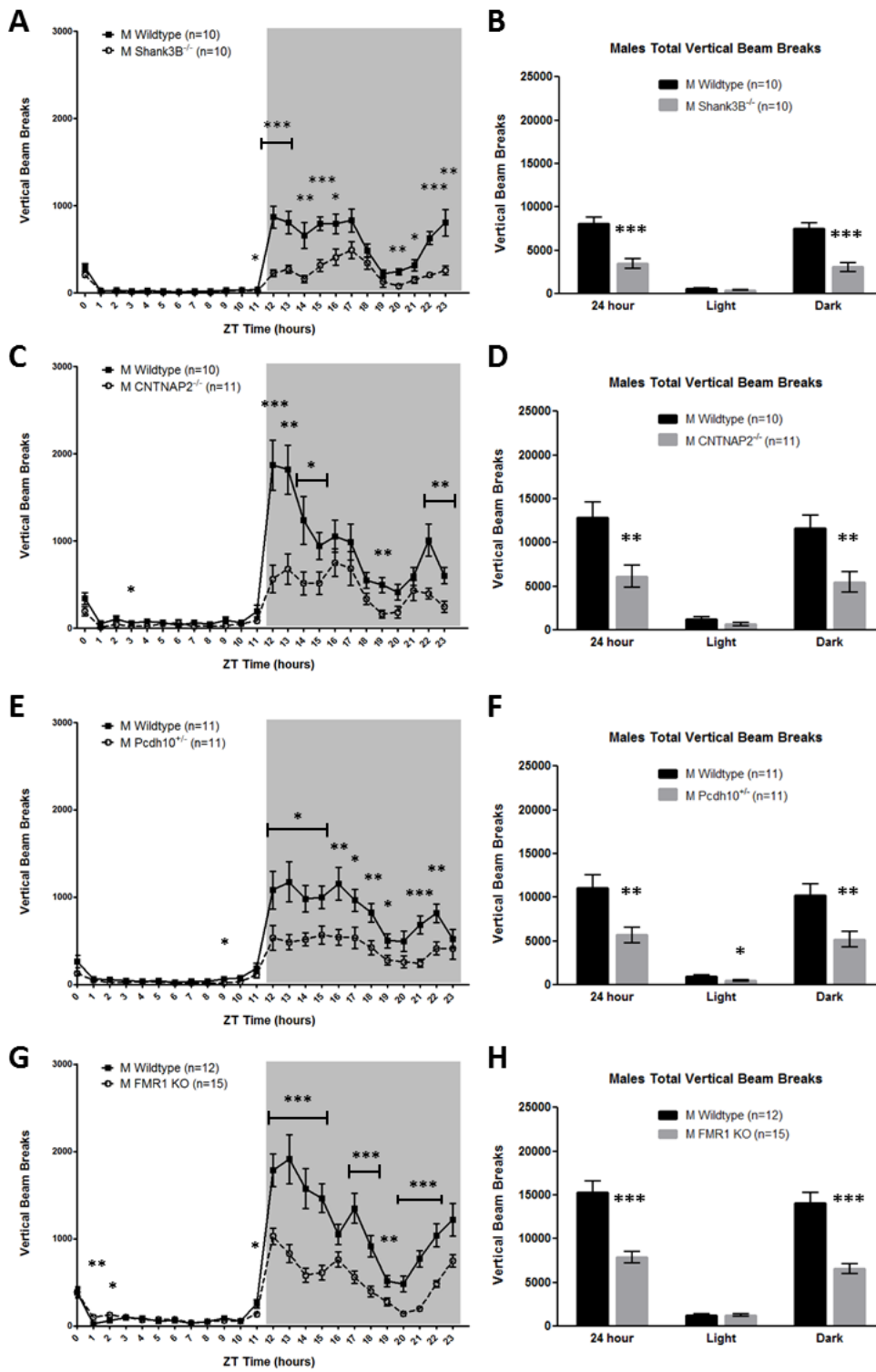




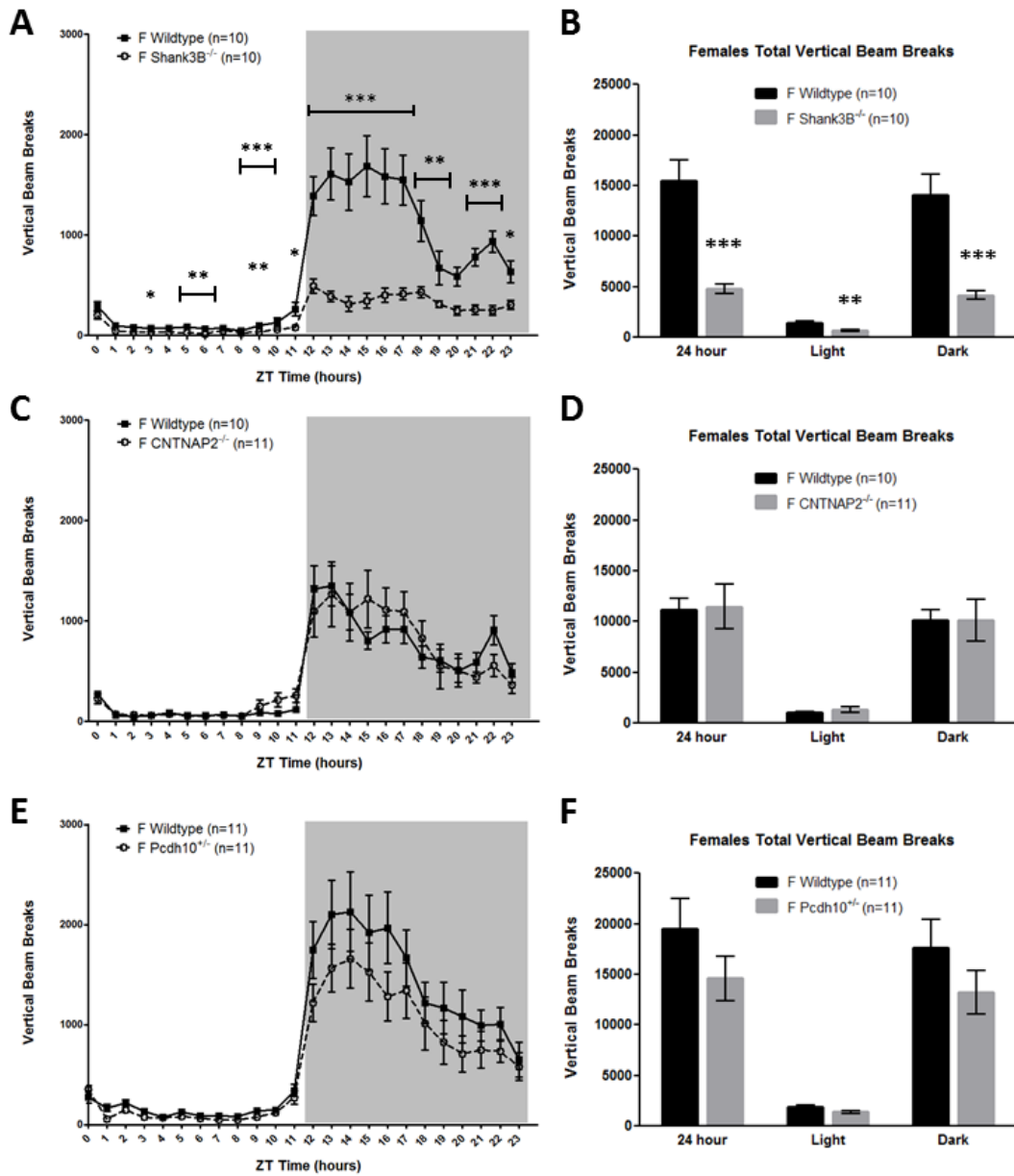
Figure 4.2



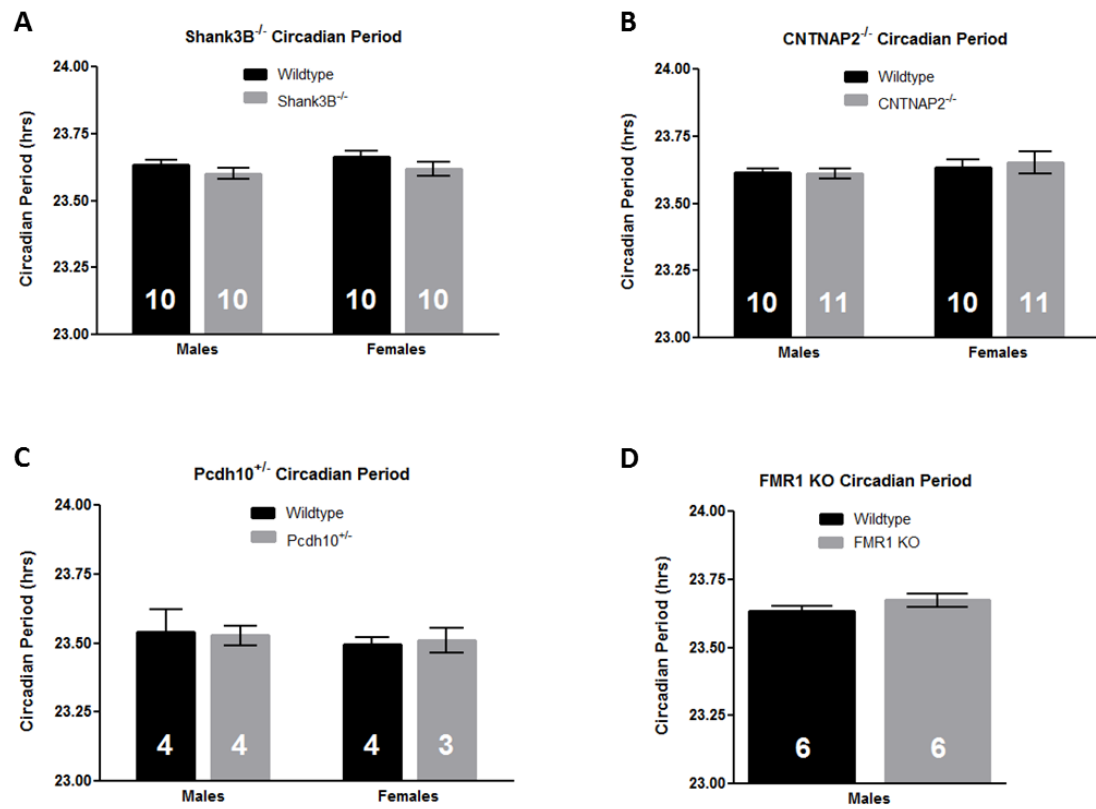
Supplementary Figure 4.1



Supplementary Figure 4.2



### Supplementary Figure 4.3



## CHAPTER 5: Conclusions and future directions

### 5.1 Conclusions

In this dissertation, I described sleep, home-cage activity, and circadian rhythms in various genetic mouse models of neurodevelopmental disorders, with a particular focus on models of ASD. In **Chapter 2**, I presented male-specific sleep reductions in the 16p11.2 del/+ mouse model of neurodevelopmental disorders, recapitulating phenotypic sex differences and total sleep time reductions commonly reported in ASD (Sivertsen et al., 2012; Robinson-Shelton & Malow, 2016; Souders et al., 2017). In **Chapter 3**, I described REM sleep reductions, a common polysomnographic finding in human ASD patients (Limoges et al., 2005; Buckley et al., 2010), and altered EEG spectra properties in the *Syngap1*<sup>+/-</sup> mouse model of intellectual disability, ASD, and epilepsy. In **Chapter 4**, I depicted the shared phenotype of home-cage hypoactivity in male mice of four different mouse models of ASD: *Shank3B*<sup>-/-</sup>, *CNTNAP2*<sup>-/-</sup>, *Pcdh10*<sup>+/-</sup>, and FMR1 KO. Interestingly, there were no home-cage activity differences in female mice for two of the four lines studied, recapitulating male-enhanced phenotypic presentation in human ASD patients in spite of the same genetic insult (Robinson et al., 2013; Werling & Geschwind, 2013; Jacquemont et al., 2014).

Accurate genetic models demonstrating face validity are important for testing potential treatment strategies and elucidating neurobiological mechanisms underlying sleep and circadian problems in neurodevelopmental disorders. Despite this, prior to this thesis work, there was a paucity of published studies investigating sleep and circadian rhythms in mouse models of neurodevelopmental disorders. The possibility of

recapitulating some of the diverse sleep and circadian problems found in human neurodevelopmental disorders in accurate rodent genetic models motivated this work. Indeed, throughout various genetic models, we discovered a male-enhanced insomnia-like phenotype (**Chapter 2**), REM sleep reductions (**Chapter 3**), sex differences (**Chapter 2** and **Chapter 4**), and a shared activity phenotype that may be indicative of thalamocorticostraial circuitry aberrations germane to the disorder (**Chapter 4**). The long-term goal in undertaking this thesis work was to provide a basis for future research to identify brain region- or cell type-specific mechanisms mediating various sleep and circadian disruptions in ASD, allowing for more targeted and tailored treatment strategies. It is our hope that the diversity of sleep, activity, and circadian phenotypes presented herein will inspire future investigations into the precise circuitries and mechanisms underlying specific sleep and circadian disruptions in human ASDs.

## **5.2 Genetics of ASD**

Evidence from twin studies, family studies, and genome-wide association studies has revealed a strong genetic component to ASD (Persico & Napolioni, 2013; Smalley et al., 1988; Weiss et al., 2009). However, ASD is an exceptionally diverse and complex spectrum of disorders, grouped together by common phenotypes such as social deficits, communication problems, and restricted or repetitive behaviors (Geschwind, 2011; Persico & Napolioni, 2013a). Rather than a few common genetic variants resulting in ASD, numerous rare genetic variants with low individual penetrance, and even the synergistic effect of many common genetic variations, can contribute to symptomology ultimately classified as ASD. Highlighting this, risk factor genes associated with ASD represent a wide array of biochemical classes and functions, including genes known to be involved in

mRNA translation, DNA methylation, chromatin remodeling, synaptic excitability, cell adhesion, growth-regulation and cell proliferation,  $\text{Ca}^{2+}$  signaling, and more (Reviewed in: Persico & Napolioni, 2013). In addition to single-gene risk factors, numerous copy number variations, including in chromosomal region 16p11.2 (**Chapter 2**), have been shown to be strongly associated with increased prevalence of neurodevelopmental disorders (Levy et al., 2011; Sanders et al., 2011). Thus, rather than treating ASD-associated behavioral symptomologies such as insomnia, hyperactivity, social impairments, and communication problems as monolithic, perhaps individualized genetics-based treatment strategies aimed at targeting specific molecular mechanisms and circuitry would be more effective. Genetics-based treatment approaches may alleviate issues such as administering psychomotor stimulants to patients with comorbid ADHD and ASD, which may help assuage hyperactivity, but is likely to exacerbate sleep problems. Discovering neurobiological mechanisms underlying phenotypic abnormalities is one of the main benefits and utilities of rodent genetic models. The disparate phenotypes observed in the different ASD mouse models investigated in this thesis work highlights the need for more genetic models of neurodevelopmental disorders capable of recapitulating the symptomologies of their human homologs. Fortunately, with recent advancements in genome engineering, generating novel transgenic mouse models is easier than ever.

### **5.3 Sex differences in ASD**

ASDs and related neurodevelopmental disorders such as ADHD are highly sex-biased. Males are 4.5 times more likely than females to be diagnosed with ASD (Werling & Geschwind, 2013; Christensen et al., 2016), and 3 times more likely to be diagnosed

with ADHD (Schneider & Eisenberg, 2006). Moreover, males often present more severe symptomologies than do females, given the same genetic insult (Robinson et al., 2013; Jacquemont et al., 2014). Reasons behind these strong sex differences in ASD and related neurodevelopmental disorders are not known. The vast majority of studies performed in rodent models of ASD do not directly compare males and females. In the only other studies to date investigating sleep in rodent models of ASD, comparisons between males and females were not made (Cusmano & Mong, 2014; Thomas et al., 2016). Thus, the male-specific sleep time reductions and wake bout duration alterations in 16p11.2 del/+ mice presented in **Chapter 2** are the first demonstrated sex differences in sleep in a rodent genetic model of autism. We also found sex differences in activity, relative to sex-matched WT littermates, in two of the four ASD-associated monogenic models studied in **Chapter 4**. Further rodent models recapitulating the sex differences observed in ASD are greatly needed in order to understand the contributing mechanisms. Thus, one hopeful future direction as a consequence of this research is the enhancement of studies directly comparing males and females when investigating rodent models of ASD and other sex-biased neurodevelopmental disorders.

#### **5.4 Targeted genetic manipulations of ASD-associated genes and CNVs**

One of the main advantages of rodent models is the capacity they have for spatially- and temporally-restricted genetic manipulation (reviewed in: Angelakos & Abel, 2015). The findings presented in this dissertation were all performed in mice expressing constitutive genetic deletion, reduction, or mutation. These results provide an exciting preliminary step in identifying sleep, activity, and circadian alterations in various mouse models. Even more exciting are the future directions of restricting genetic manipulation in



time and space. For example, bout distribution analysis in **Chapter 2** suggests that 16p11.2 del/+ male mice may have an overactive wake-promoting system, maintaining unusually long bouts of continuous wakefulness. This data, in concert with the robust hyperactivity observations, and findings from other labs in 16p11.2 del/+ mice, suggests irregular dopaminergic signaling (Portmann et al., 2014; Grissom et al., 2017). Therefore, one interesting potential avenue of future investigation is to study sleep and activity in 16p11.2 hemideletion restricted to only dopamine D1- or dopamine D2-receptor expressing cells. As another example, in **Chapter 3**, I show REM sleep reductions and elevated baseline NREM delta in *Syngap1*<sup>+/-</sup> mice, relative to WT. REM sleep is thought to be generated by reciprocal interactions of brainstem nuclei (Sastre et al., 1996; Boissard et al., 2002; Lu et al., 2006; Luppi et al., 2006). Conversely, two different delta rhythms are known to exist: one generated by T-Type Ca<sup>2+</sup> channels and Ca<sup>2+</sup>-dependent small-conductance (SK)-type K<sup>+</sup> channels in the thalamic reticular nucleus (Cueni et al., 2008; Lee et al., 2004), and another generated locally in the cortex (Steriade et al., 1993; Sanchez-Vives & McCormick, 2000; Timofeev et al., 2000). Thus, using mouse Cre lines restricting SYNGAP1 reduction to the cortex, or viral Cre delivery to brainstem nuclei in *Syngap1* floxed mice, may prove extremely useful in identifying the particular neural circuits and mechanisms underlying the sleep phenotypes observed in *Syngap1*<sup>+/-</sup> mice.

## **5.5 Reversal of phenotypes and impact of development**

Another potential future direction based upon the findings presented in this dissertation is the reversal of phenotypes using pharmacological or genetic manipulation. Rodent models provide the ideal opportunity to test potential treatment strategies before proceeding to clinical trials in humans. For instance, our findings in **Chapter 2** suggest

that dopamine subtype-specific drugs may ameliorate the hyperactivity and overactive wake system of 16p11.2 del/+ mice. One potential drug to test might be risperidone, a dopamine D2 antagonist that is already approved for use in human ASD patients. In addition to pharmacological treatment, genetic manipulations could be employed in an attempt to reverse some of the sleep, activity, and circadian alterations presented in this dissertation. Viral-mediated enhancement of SYGNAP1, Shank3B, CNTNAP2, or Pcdh10, or inducible Cre recombination using a FLEEx switch (Schnütgen et al., 2003; Atasoy et al., 2008), may normalize some of the phenotypes observed in these mouse models.

One limitation of these studies is that all of the experiments were performed in adult mice, but ASD is a developmental disorder. Technical limitations prevented us from implanting and recording sleep in juvenile mice. Future studies employing innovative technologies to characterize sleep and activity across development would be beneficial. For the time being, adult-restricted restoration of these ASD-associated genes or chromosomal regions using temporally-restricted promoters, tamoxifen-inducible Cre, or viral-mediated FLEEx Cre technologies may provide useful insight into the potential reversibility of phenotypes beyond a critical period of neural development. For example, do the activity alterations observed in the seven mouse models studied in this dissertation persist following genetic restoration in adult mice? If so, the phenotype may not be reversible beyond a certain developmental time point.

## **5.6 Summary and future directions**

In summary, the work presented in this thesis highlights novel sleep, activity, and circadian alterations in multiple genetic mouse models of human neurodevelopmental disorders. In some instances, the phenotypes observed were male-specific, recapitulating

sex-biases observed in human ASD. These studies provide a foundation for modeling various sleep problems commonly reported in individuals diagnosed with neurodevelopmental disorders. Future directions involving more spatially- and temporally-restricted genetic mutation are likely to provide further insight into the neurobiological mechanisms mediating the phenotypes reported in this dissertation. Another potential benefit of the findings from these studies is the possibility to exploit these mouse models to test pharmacologic interventions and potential treatment strategies for the amelioration of these phenotypes. Finally, these studies emphasize the utility of transgenic mice for modeling human disorders. An important implication of this work is the continued development of novel genetic models, and the insistence on performing well-controlled and direct comparisons between male and female mice in models of sex-biased disorders.

**Author Contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Ted Abel.

## APPENDIX: Circadian gene expression alterations and prolonged free-running period in CBP<sup>Kix/Kix</sup> mice

### Abstract

The molecular circadian clock is controlled by core clock genes participating in transcription-translation feedback loops to maintain precise temporal rhythms. The transcription factor cAMP response element-binding protein (CREB) and the CREB-binding protein (CBP) are both known regulators of Period (Per) transcription and circadian rhythmicity. CBP overexpression enhances CLOCK/BMAL1-mediated Per1 activation, while CBP knockdown decreases circadian clock induction and oscillation. Despite this, the mechanisms underlying the relationship between CREB, CBP, and circadian rhythms are not fully understood. We performed RNA sequencing in the hippocampus of mice carrying an inactivating triple point mutation in the CREB-binding (Kix) domain of CBP (CBP<sup>Kix/Kix</sup> mice), which blocks phospho-CREB binding to CBP. We found altered expression of the circadian clock mediator genes *Gm129* (*Chrono*) and *Dbp* in CBP<sup>Kix/Kix</sup> mice, which we validated with quantitative PCR. Activity monitoring of CBP<sup>Kix/Kix</sup> mice across the diurnal cycle in 12 hour: 12 hour light:dark revealed delayed activity onset as well as delayed peak activity in both male and female CBP<sup>Kix/Kix</sup> mice compared to sex-matched wildtype littermates. Similar to prolonged circadian periods found in *Chrono* knockout mice, CBP<sup>Kix/Kix</sup> mice exhibited a 23-minute increase in free-running circadian period relative to wildtype littermates; however phase shifting in response to 15-minute light pulses at CT14 and CT22 did not differ between CBP<sup>Kix/Kix</sup> mice and controls. Together, these studies help to further elucidate the molecular machinery regulating the circadian clock by highlighting a novel role for CREB-mediated transcription through CBP in maintaining accurate circadian rhythmicity.

## Introduction

Nearly all organisms exhibit intrinsic circadian rhythms, which are entrained by environmental stimuli to the 24-hour diurnal cycle of the rotating earth (Jay C Dunlap, 1999; Edgar et al., 2012). In mammals, light entering the retina sets off a molecular cascade via the retinohypothalamic tract, leading to the master circadian clock in the suprachiasmatic nucleus (SCN) (Moore & Eichler, 1972; Stephan & Zucker, 1972.; Welsh et al., 1995). These circadian rhythms are maintained via the bidirectional transcription-translation activity of core clock genes. Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) heterodimerizes with CLOCK in the nucleus and binds to E-box enhancer sequences in the promoters of numerous genes, including the transcriptional repressors Period (PER) and Cryptochrome (CRY), initiating their transcription. PER and CRY in turn inhibit transcription of BMAL1 and CLOCK (Gekakis et al., 1998; Reppert & Weaver, 2002), creating a feedback loop controlling circadian rhythmicity. In addition to these core genes, many other transcriptional activators and repressors have been identified as important circadian regulatory elements (see Buhr & Takahashi, 2013; Golombek & Rosenstein, 2010; Partch et al., 2014 for recent reviews).

One molecule long known to play a critical role in circadian regulation is the transcription factor cAMP response element-binding protein (CREB). Phosphorylated CREB levels oscillate in a circadian manner in the SCN, and light pulses during the night rapidly induce CREB phosphorylation and cAMP response element (CRE)-mediated gene expression (Ginty et al., 1993; Obrietan et al., 1999; von Gall et al., 1998). Both the *Per1* and *Per2* promoters contain CREs capable of binding p-CREB in SCN extracts, and mutation of the CRE in the *Per1* promoter abolishes the promoter's responsiveness to various forms of cellular induction (Travnickova-Bendova et al., 2002). Moreover, a S142A

mutation of CREB in mice, preventing phosphorylation at Ser142, attenuates light-induced behavioral phase shifts and significantly reduces *Per1* expression (Gau et al., 2002).

In addition to CREB, the CREB-binding protein (CBP) is a known circadian regulator (Hung et al., 2007). CBP is recruited by and heterodimerizes with the CLOCK/BMAL1 complex, directly interacting with BMAL1 (Hosoda et al., 2009; Takahata et al., 2000). CBP overexpression increases CLOCK/BMAL1-mediated *Per1* transcription, and this is dependent on functional E-box elements within the *Per1* promoter. Conversely, siRNA knockdown of CBP significantly diminishes circadian oscillation in cultured cells, whereas knockdown of p300 or CREB do not (Y. Lee et al., 2010). Interestingly, CBP has been shown to bind to E-boxes at both CT4 and CT16, suggesting that it may have disparate transcriptional co-activator and co-repressor roles. In fact, CBP also interacts with the transcriptional repressor PER2 (Nobuya Koike et al., 2012).

Although CREB and CBP have both been shown to be key regulators of circadian rhythms, much is unknown about the underlying mechanisms. To investigate the direct relationship between CREB, CBP, and circadian rhythms, we utilized mice expressing an inactivating triple point mutation in the p-CREB-interacting (Kix) domain of CBP (CBP<sup>Kix/Kix</sup> mice) (Kasper et al., 2002), which blocks phospho-CREB-mediated transcription through CBP (Kwok et al., 1994). Previously, CBP<sup>Kix/Kix</sup> mice have been shown to have disrupted long-term memory (Wood et al., 2006; Stefanko et al., 2009); however their circadian behavior has not been studied. In the present study, we studied the transcriptional profile as well as the circadian behavior of CBP<sup>Kix/Kix</sup> mice. Through RNA-seq and qPCR, we found altered expression of circadian regulatory genes in CBP<sup>Kix/Kix</sup> mice relative to WT littermates. Activity monitoring in 12 hour: 12 hour light:dark (12h:12h LD) revealed temporally delayed circadian behavior in CBP<sup>Kix/Kix</sup> mice compared to WT controls. In

constant darkness (DD), CBP<sup>Kix/Kix</sup> mice exhibited a lengthened free-running circadian period relative to WT. Together, this data highlights a novel mechanism by which CREB-mediated transcription through CBP impacts circadian rhythmicity, further advancing our knowledge of the contributions of these important circadian regulators to the maintenance and resetting of circadian rhythms.

## **Materials and Methods**

### *Animals*

CBP<sup>Kix/Kix</sup> mice were previously generated with an inactivating triple-point mutation of Tyr650Ala, Ala654Gln, and Tyr658Ala of CBP, preventing CREB binding to the Kix domain of CBP (Kasper et al., 2002b). CBP<sup>Kix/Kix</sup> mice were backcrossed on a C57Bl/6 background for over ten generations, and heterozygous (CBP<sup>Kix/+</sup>) breeders were used for the generation of experimental animals, with sibling wildtype littermates serving as controls. Mice were group housed in cages of 3-5 littermates and were maintained on a 12h:12h LD cycle with light onset at 7:00am, except where indicated otherwise. Separate animals were used for RNA-seq/qPCR experiments and the activity monitoring/circadian behavior experiments. All mice were between 3 and 5 months old at the time of tissue collection or the beginning of activity monitoring experimentation. One mouse was excluded from behavioral experiments due to unusually high levels of activity (>2.5 standard deviations above the group mean). Food and water was provided *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and conducted in accordance with National Institutes of Health guidelines.



### *Fear Conditioning*

Contextual fear conditioning was performed as previously described (Vecsey et al., 2007) with mice handled 1 minute per day for 3 consecutive days prior to fear conditioning training. On the training day, mice received a single 2s, 1.5mA footshock, culminating 2.5min after placement into the chambers. 30s after the footshock, mice were removed from the chambers and returned to their home-cages. A separate group serving as home-cage controls was handled, but did not receive fear conditioning training.

### *RNA Isolation*

30 minutes after fear conditioning or home-cage control, mice were sacrificed between ZT3.5-ZT4 and hippocampi were rapidly dissected into RNAlater (Thermo Fisher Scientific, Waltham, MA) and immediately frozen on dry ice. Tissue was homogenized and lysed in Qiazol and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Trace genomic DNA was removed by treating with RNase-Free DNase (Qiagen) off-column. Subsequently, RNA was precipitated in ethanol and resuspended in nuclease free water.

### *RNA Sequencing*

Library preparation was performed using the TruSeq RNA Sample Prep Kit with polyA selection (Illumina San Diego, CA) according to manufacturer's instructions. Samples were sequenced at the PGFI Sequencing Core at the University of Pennsylvania using the HiSeq 2000 (Illumina San Diego, CA) and 100 bp paired-end reads. The quality of the sequencing data was checked using FASTQC (Andrews, 2010) and all samples had Phred quality scores > 30. Sequencing files were aligned to the

most recent mouse genome, mm10, using GSNAP/GMAP (Wu & Watanabe, 2005; Wu & Nacu, 2010) (<http://research-pub.gene.com/gmap/>). Only the concordant and unique reads were then annotated and reads were counted using the R program, featureCounts (Liao et al., 2014) (<http://bioinf.wehi.edu.au/featureCounts/>). Removal of Unwanted Variation (RUV) sequencing was then implemented to control for unknown variables, as previously described (Risso et al., 2014; Peixoto et al., 2015; Gerstner et al., 2016; Poplawski et al., 2016). Normalized read counts were compared between CBP<sup>Kix/Kix</sup> home-cage samples and CBP<sup>Kix/Kix</sup> fear-conditioned samples.

#### *cDNA preparation and qPCR*

RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and cDNA was prepared using a RETROscript kit (Ambion, Austin, TX) from 1 µg total RNA. Real-time PCR (qPCR) was performed with ViiA 7 PCR system (Applied Biosystems, Waltham, Massachusetts) using 3 housekeeper genes for normalization (ActinG, Tuba4a, and Hprt). Differential gene expression was quantified using a  $\Delta\Delta C_t$  method. The primers used for the analysis were:

Gm129 FW 5' CATCCTATGTCCGCCTGTCC 3'

Gm129 Rev 5' ACTGGGGGATGGCTAGTCAT 3'

Dbp FW 5' CCTTTGAACCTGATCCCGCT 3'

Dbp Rev 5' CTCTGAGAAGCGGTGTCTCC 3'

Hprt5 FW 5'-TTGCTGACCTGCTGGATTACA-3'

Hprt5 Rev 5'-CCCCGTTGACTGATCATTACA-3'

Tuba4a FW 5'-ATGCGCGAGTGCATTTTCAG-3'

Tuba4a Rev 5'-CACCAATGGTCTTATCGCTGG-3'

ActinG FW 5'-CCGATCGCAATGGAAGAAG-3'

ActinG Rev 5'-CGTATGAGTCTTTCTGGCCCA-3'

### *Activity Monitoring*

Activity monitoring was performed as previously described in a separate group of mice from the RNA-seq / qPCR experiments (Angelakos et al., 2016). Briefly, mice were individually housed inside light- and noise-attenuating chambers (22" x 16" x 19", Med Associates, St. Albans, VT) equipped with a 250 lux light source (80 lux at cage floor) and fan for ventilation. Each cage was placed within a system of infrared beams spaced 0.5 inches apart, which provided two horizontal infrared grids at 0.75" and 2.75" above the cage floor. Mice were allowed to acclimate to the activity monitoring chambers for one week before experimentation. Following acclimation, infrared beam break counts were acquired at 10 second intervals for 7 days on a 12 hour light / 12 hour dark (12h:12h LD) schedule to measure both horizontal and vertical (rearing) activity across the entire diurnal cycle. Activity counts were binned into 1-hour intervals and averaged over the 7 days. After the final day of activity monitoring in 12h:12h LD, lights were switched off and animals were allowed to free-run in 24-hour constant darkness (DD) for 4 weeks, with activity counts compiled every minute. Circadian period ( $\tau$ ) was calculated from Day 2 until the end of DD using ClockLab software (Actimetrics).

### *Light Pulses*

Following 4 weeks of 24-hour constant darkness, one cohort of mice (n=8 WT and n=5 CBP<sup>Kix/Kix</sup>) received a 15-minute phase delaying 250 lux (80 lux at cage floor) light

pulse at CT14. Mice were allowed to recover for 8 days in 24-hour constant darkness and tau was recalculated. After the 8 days in continuous darkness, a second 15-minute phase advancing light pulse was given at CT22. 8 days later, tau was recalculated. The phase angles of activity delay and advancement were calculated using ClockLab software (Actimetrics).

### *Statistics*

All statistical analysis was performed using SPSS for Windows (V. 24.0). Two-way ANOVAs were performed for qPCR studies with genotype and fear condition as the independent variables, as well as for circadian tau analysis in DD, with genotype and sex as the independent variables. Mixed-Design ANOVAs were utilized for beam-break activity analysis, with genotype (CBP<sup>Kix/Kix</sup> or WT) as the between-subjects factor and time as the within-subjects factor. Post hoc multiple comparisons were performed using Bonferroni's adjustment for multiple comparisons. Multivariate ANOVAs (MANOVA) was used to analyze the activity counts in the light and dark phases, with alpha corrected for multiple ANOVAs and set at  $\alpha = 0.05/2$ . For total activity over the 24-hour day and light pulse analysis, Student's t-test was applied.

## **Results**

### ***CBP<sup>Kix/Kix</sup> mice have decreased Gm129 and Dbp expression at ZT4***

RNA-sequencing was performed in the hippocampi of CBP<sup>Kix/Kix</sup> mice and WT littermates. Because CBP<sup>Kix/Kix</sup> mice have previously been shown to have contextual fear memory deficits (Wood et al., 2006), we sequenced RNA isolated from the hippocampi of CBP<sup>Kix/Kix</sup> and WT mice sacrificed 30-minutes after contextual fear conditioning as well as

from mice that were undisturbed in the home-cage, resulting in four experimental groups (Table A.1 and Fig. A.1). Removal of Unwanted Variation (RUV) normalization was performed as previously described (Risso et al., 2014; Peixoto et al., 2015; Gerstner et al., 2016; Poplawski et al., 2016). Comparing the home-cage housed CBP<sup>Kix/Kix</sup> mice to home-cage housed WT mice, we identified at FDR < 0.05, 127 genes downregulated (Table A.2) and 62 genes upregulated (Table A.3) in CBP<sup>Kix/Kix</sup> mice relative to WT. Comparing the two genotypes after contextual fear conditioning, we identified 358 downregulated genes (Table A.4) and 125 upregulated genes (Table A.5) in CBP<sup>Kix/Kix</sup> mice relative to WT. Interestingly, two of the genes downregulated in CBP<sup>Kix/Kix</sup> mice in both the home-cage and fear conditioning groups, gene model 129 (*Gm129/Chrono*) and D site albumin promoter binding protein (*Dbp*), are known regulators of the mammalian circadian clock. *Chrono* was recently identified through ChIP-seq and machine learning as a repressor of CLOCK/BMAL1 function through direct binding to the CLOCK/BMAL1 complex (Anafi, Lee, Sato, Venkataraman, Ramanathan, Kavakli, Hughes, Baggs, et al., 2014; Annayev et al., 2014a; Goriki et al., 2014). *Dbp* is highly rhythmic in the SCN during both light-dark and dark-dark conditions (Lopez-Molina et al., 1997), binds to the *mPer1* promoter, increasing *mPer1* transcription, and is transcriptionally regulated by the core circadian CLOCK/BMAL1 complex (Yamaguchi et al., 2000).

The differential expression of *Chrono* and *Dbp* was validated with qPCR in the same cohort of mice used for RNA-sequencing. In line with the RNA-sequencing results, *Chrono* and *Dbp* RNA levels were decreased in CBP<sup>Kix/Kix</sup> mice relative to WT mice in both home-cage and fear conditioned conditions at ZT4 (main effects of genotype, *Chrono*:  $F(1,12) = 15.39$ ,  $p=0.002$ ; *Dbp*:  $F(1,12) = 6.23$ ,  $p=0.028$ , Fig. A.2). There was no main

effect of fear conditioning (Chrono:  $F(1,12) = 0.04$ ,  $p=0.85$ ; Dbp:  $F(1,12) = 0.08$ ,  $p=0.78$ ) nor a genotype\*fear condition interaction (Chrono:  $F(1,12) = 0.38$ ,  $p=0.55$ ; Dbp:  $F(1,12) = 2.28$ ,  $p=0.16$ ). Thus, altered *Chrono* and *Dbp* expression is an intrinsic property of  $CBP^{Kix/Kix}$  mice.

### ***$CBP^{Kix/Kix}$ mice have delayed activity onset and peak activity in the light-dark cycle***

Because  $CBP^{Kix/Kix}$  mice have altered expression of genes known to impact the circadian clock, we sought to behaviorally profile the activity patterns of  $CBP^{Kix/Kix}$  mice and WT controls. Home-cage activity was first quantified via infrared beam breaks over one week of continuous monitoring in 12h:12h LD, as previously described (Angelakos et al., 2016). Male and female activity was analyzed separately due to the known sex-differences in mouse activity (Nishi et al., 2010; Angelakos et al., 2016). Mixed Design ANOVAs revealed a significant time\*genotype interaction between male  $CBP^{Kix/Kix}$  and WT mice ( $F(23,368) = 6.13$ ,  $p = 0.002$ ; Fig. A.3a). *Post hoc* comparisons indicated that  $CBP^{Kix/Kix}$  males had significantly lower activity during the first hour of the dark(active) phase ( $t(16) = 19.54$ ,  $p < 0.001$ ) and reached peak activity levels 3 hours later than WT males. There was no difference in total activity across the 24-hour day (Student's t-test,  $t(16) = 1.16$ ,  $p= 0.307$ ; Fig. A.3b), nor during the light or dark phases (MANOVA,  $F(2,15) = 2.47$ ,  $p = 0.118$ ; Fig. A.3b) between  $CBP^{Kix/Kix}$  and WT males. Likewise, there was also a significant time\*genotype interaction between female  $CBP^{Kix/Kix}$  mice and WT littermates ( $F(2,23) = 7.95$ ,  $p < 0.001$ ; Fig. A.3c).  $CBP^{Kix/Kix}$  females had decreased activity relative to WT littermates each of the first 3 hours of the dark(active) phase (all  $p < 0.01$ ), and also reached peak activity of the initial activity wave 3 hours later than WT females. However, unlike the males,  $CBP^{Kix/Kix}$  females reached maximum activity levels following their siesta

at ZT21. There were no differences in activity counts between CBP<sup>Kix/Kix</sup> females and WT littermates over the 24-hour day (Student's t-test:  $t(10) = 1.11$ ,  $p = 0.316$ ; Fig. A.3d), nor in the light or dark phases (MANOVA,  $F(2,9) = 0.62$ ,  $p = 0.56$ ; Fig. A.3d). These data indicate that in comparison to WT mice, CBP<sup>Kix/Kix</sup> mice have delayed activity patterns in 12h:12h LD while maintaining normal total locomotor activity.

### ***CBP<sup>Kix/Kix</sup> mice have increased circadian tau in constant darkness***

Given the temporally shifted activity patterns of CBP<sup>Kix/Kix</sup> mice observed in 12h:12h LD, we next free-ran CBP<sup>Kix/Kix</sup> and WT mice in constant darkness for 4 weeks to gauge free-running circadian period. There was no effect of sex on tau ( $p=0.33$ ), nor a sex\*genotype interaction (Two-way ANOVA,  $p = 0.78$ ; Fig. A.4a). Thus, sexes were combined for analysis of circadian tau. On average, CBP<sup>Kix/Kix</sup> mice had a circadian period ( $24.05 \pm 0.03$  hrs) that was ~22.9 minutes longer than that of their WT counterparts ( $23.66 \pm 0.01$  hrs) (main effect of genotype,  $F(1,26) = 126.183$ ,  $p = 0.000000000002$ ; Fig. A.4b, see Fig. A.4c and A.4d for representative traces closest to the group means for WT and CBP<sup>Kix/Kix</sup>, respectively). This ~30 minute shift in circadian tau is roughly similar to the shifts observed in knockouts of core circadian clock genes, including *CLOCK* (DeBruyne et al., 2006), *Per1* (Cermakian et al., 2001), *Per3* (Shearman et al., 2000), *Nr1d1* (Preitner, Damiola, Lopez-Molina, et al., 2002), *Dbp* (Lopez-Molina et al., 1997), *Rora* (Sato et al., 2004), *Rorb* (André et al., 1998), and *Npas2* (Dudley et al., 2003) [summarized in (Ko & Takahashi, 2006)]. Thus, the Kix domain of CBP is an integral modulator of circadian timing.

### ***CBP<sup>Kix/Kix</sup> mice have normal phase shifts to light pulses***

To further probe the role of the Kix domain of CBP in the circadian clock, after 4 weeks in continuous darkness, one cohort of CBP<sup>Kix/Kix</sup> mice and WT controls was given a 15-minute, 250 lux (80 lux at cage floor) phase delaying light pulse at CT14 for maximal phase delay (Jud et al., 2005). Mice were allowed to again free-run in constant darkness for 8 days before receiving another light pulse at CT22. Circadian tau was recalculated and the phase shift in hours was calculated for each animal. There were no differences in response to the delaying (CT14) or advancing (CT22) phase shifts between CBP<sup>Kix/Kix</sup> mice and WT controls (Student's t-test, phase delaying: (t(11) = 1.15, p = 0.27; phase advancing: (t(11) = 0.93, p = 0.37; Fig. A.5a). This data suggests that p-CREB-mediated transcription through CBP is not necessary for circadian clock resetting, but rather is a modulator and plays a role in ensuring accurate timing of circadian transcriptional oscillations.

## Discussion

In this study, we investigated gene transcription and the circadian behavioral profile of mice deficient for CREB-mediated transcription through CBP. RNA-seq and qPCR validation revealed altered expression of the circadian regulatory genes *Chrono* and *Dbp* in CBP<sup>Kix/Kix</sup> mice relative to WT littermates. In 12h:12h LD diurnal activity monitoring, CBP<sup>Kix/Kix</sup> mice have delayed activity onset and peak activity compared to sex-matched controls. In constant darkness, CBP<sup>Kix/Kix</sup> mice exhibit prolonged free-running circadian periods. Despite the temporally delayed circadian activity of CBP<sup>Kix/Kix</sup> mice, they respond normally to clock resetting light pulses, suggesting that CREB-mediated transcription through the Kix domain of CBP is necessary for modulating the circadian clock, but is not required for entrainment. Altogether, this data identifies a novel



mechanism for the regulation of circadian timing through CBP-dependent CREB-mediated transcription.

The CBP is a large 265 kDa protein containing multiple interaction domains (nuclear hormone receptor binding domain, zinc fingers, Kix domain, bromodomain, histone acetyltransferase domain, glutamine-rich (Q) domain) and is believed to interact with hundreds of transcription factors (Goodman & Smolik, 2000; Vo & Goodman, 2001; Kalkhoven, 2004). Our data help pin down a specific mechanism by which CBP can regulate circadian gene transcription and behavior. The relationship between CREB, CBP, and circadian rhythms is likely complex. CREB phosphorylation at both Ser-133 and Ser-142 is circadian regulated and driven by light (Ginty et al., 1993; C von Gall et al., 1998; Obrietan et al., 1999; Gau et al., 2002); however Ser-133 and Ser-142 phosphorylation exhibit opposing effects on CBP transcriptional activity. Phosphorylation of Ser-133 promotes CREB interaction and transcription via the Kix domain of CBP, while Ser-142 phosphorylation inhibits this interaction (D. Parker et al., 1998). Since CBP<sup>Kix/Kix</sup> abolishes transcription through the Kix domain of CBP, mice expressing an inactivating mutation of Ser-133, or mice with a constitutively phosphorylated Ser-142, may be expected to exhibit circadian phenotypes similar to those observed in CBP<sup>Kix/Kix</sup> mice.

The reduction of *Chrono* in CBP<sup>Kix/Kix</sup> mice relative to WT is particularly interesting considering recent findings. *Chrono* is a transcriptional repressor of the CLOCK/BMAL1 complex, acting phenotypically similarly to *Cry2*, albeit via distinct repressive mechanisms (Anafi et al., 2014; Annayev et al., 2014b; Goriki et al., 2014). Interestingly, *Chrono* knockout mice display an ~25 minute lengthening of free-running circadian period (Anafi et al., 2014), which is almost identical to the ~22.9 minute prolonged circadian period found in CBP<sup>Kix/Kix</sup> mice relative to WT. Moreover, *Chrono* binds to the E-box of *Dbp*

reducing *Dbp* promoter activity, and in *Chrono*<sup>-/-</sup> mice, the peak expression of *Dbp* is extended (Annayev et al., 2014; Goriki et al., 2014). Since *Dbp* is a pure E-box gene indicative of CLOCK/BMAL1 activity, the lengthening of peak *Dbp* expression through a *Chrono*-dependent mechanism may explain the altered *Dbp* levels and circadian behavioral delays in *CBP*<sup>Kix/Kix</sup> mice.

Interestingly, Anafi et al. recently showed that *Chrono* abrogates the binding of CBP to the CLOCK/BMAL1 complex, a very likely mechanism of *Chrono*'s repressive effects on the circadian clock (Anafi et al., 2014). Our data in the present study shows that mutation of the Kix domain of CBP in turn alters *Chrono* expression (Table 1, Figure 1). The mechanism underlying this bidirectional relationship between CBP (specifically CREB-mediated transcription through CBP) and *Chrono* requires further study. It should be noted that both CBP and *Chrono* also interact with the transcriptional repressor Per2 (Koike et al., 2012; Anafi et al., 2014; Annayev et al., 2014), indicating that the relationship between *Chrono* and *CBP*<sup>Kix/Kix</sup> may be multifaceted, and may exert different and possibly opposing effects depending on the time of the circadian cycle.

Some limitations exist in our study of the circadian behavior and gene expression of *CBP*<sup>Kix/Kix</sup> mice. Due to *CBP*<sup>Kix/Kix</sup> mice being poor breeders, it was not possible to generate sufficient mice to obtain a transcriptional profile of *CBP*<sup>Kix/Kix</sup> mice at numerous time points across the circadian cycle. Additionally, we performed RNA-sequencing in the hippocampus of *CBP*<sup>Kix/Kix</sup> mice given their well-established deficits in hippocampus-dependent long-term memory (Wood et al., 2006; Stefanko et al., 2009), however the circadian phenotypes described herein are likely to be mediated by the SCN. Finally, *CBP*<sup>Kix/Kix</sup> mice are full body knock-ins expressing mutant CBP throughout development. Our findings describe a previously unknown mechanism by which phospho-CREB-

mediated transcription through CBP modulates circadian gene transcription and behavior. Future studies quantifying *Chrono* and *Dbp* expression across the circadian cycle in mice expressing regionally-restricted adult knock-in of CBP<sup>Kix/Kix</sup> will likely shed further insight into the mechanisms underlying the relationship between CREB, CBP, and circadian rhythms.

**Author Contributions:** This chapter was written by Christopher Angelakos with input and suggestions from Ted Abel. Christopher Angelakos, John Hogenesch, and Ted Abel designed the study. Shane Poplawski and Marie Gaine performed RNA-sequencing and analysis. Snehajyoti Chatterjee performed and analyzed qPCR experiments. Christopher Angelakos, Shane Poplawski, and Giulia Porcari conducted behavioral experiments. Christopher Angelakos analyzed behavioral data and performed all statistical analysis.

## Figure Legends

**Table A.1. CBP<sup>Kix/Kix</sup> gene expression experimental groups.** Table outlining the four experimental groups utilized in gene expression studies and their respective manipulations.

**Table A.2. Home-cage RNA-seq downregulated genes in CBP<sup>Kix/Kix</sup> mice.** Table of all downregulated genes (FDR < 0.05) in the hippocampus of CBP<sup>Kix/Kix</sup> mice, relative to WT controls, in the home-cage (sorted by FDR). Circadian genes *Chrono* and *Dbp* are among the genes most significantly downregulated in CBP<sup>Kix/Kix</sup> mice relative to wildtype littermates (FDR<0.05). Abbreviations: logFC = log<sub>2</sub> fold change, logCPM = log<sub>2</sub> counts per million reads, LR = likelihood ratio, FDR = false discovery rate

**Table A.3. Home-cage RNA-seq upregulated genes in CBP<sup>Kix/Kix</sup> mice.** Table of all upregulated genes (FDR < 0.05) in the hippocampus of CBP<sup>Kix/Kix</sup> mice, relative to WT controls, in the home-cage (sorted by FDR). Abbreviations: logFC = log<sub>2</sub> fold change, logCPM = log<sub>2</sub> counts per million reads, LR = likelihood ratio, FDR = false discovery rate

**Table A.4. Fear conditioned RNA-seq downregulated genes in CBP<sup>Kix/Kix</sup> mice.** Table of all downregulated genes (FDR < 0.05) in the hippocampus of CBP<sup>Kix/Kix</sup> mice, relative to WT controls, after fear conditioning (sorted by FDR). Circadian genes *Chrono* and *Dbp* are among the genes most significantly downregulated in CBP<sup>Kix/Kix</sup> mice relative to wildtype littermates (FDR<0.05). Abbreviations: logFC = log<sub>2</sub> fold change, logCPM = log<sub>2</sub> counts per million reads, LR = likelihood ratio, FDR = false discovery rate

**Table A.5. Fear conditioned RNA-seq upregulated genes in CBP<sup>Kix/Kix</sup> mice.** Table of all

upregulated genes (FDR < 0.05) in the hippocampus of CBP<sup>Kix/Kix</sup> mice, relative to WT controls, after fear conditioning (sorted by FDR). Abbreviations: logFC = log<sub>2</sub> fold change, logCPM = log<sub>2</sub> counts per million reads, LR = likelihood ratio, FDR = false discovery rate

**Figure A.1. CBP<sup>Kix/Kix</sup> gene expression experimental setup.** Schematic of experimental design for gene expression studies.

**Figure A.2. Altered expression of circadian regulatory genes in CBP<sup>Kix/Kix</sup> mice.** CBP<sup>Kix/Kix</sup> mice have decreased *Chrono* and *Dbp* RNA levels in the hippocampus relative to WT at ZT3-4. \*p<0.05, \*\*p<0.01

**Figure A.3. Altered diurnal rhythms in CBP<sup>Kix/Kix</sup> mice.** **A)** CBP<sup>Kix/Kix</sup> males have delayed activity onset and peak activity in 12h:12h LD compared to wildtype littermates, without exhibiting differences in total ambulation **(B)**. **C)** CBP<sup>Kix/Kix</sup> females also have delayed diurnal activity patterns compared to wildtype (left), while total movement is unaltered between CBP<sup>Kix/Kix</sup> females and wildtype controls **(D)**. \*p<0.05, \*\*p<0.01. \*\*\*p<0.001

**Figure A.4. Lengthened circadian Tau in CBP<sup>Kix/Kix</sup> mice relative to WT.** **A,B)** CBP<sup>Kix/Kix</sup> males (A) and females (B) have prolonged free-running circadian period in constant darkness compared to wildtype mice. This parallels observations in *Chrono*<sup>-/-</sup> mice (Anafi et al., 2014). **C,D)** Representative actograms after 1 month of continuous darkness for wildtype (C) and CBP<sup>Kix/Kix</sup> mice (D). \*\*\*p<0.001, # p=0.00000000002

**Figure A.5.  $CBP^{Kix/Kix}$  mice have normal circadian entrainment.** A)  $CBP^{Kix/Kix}$  and WT mice display normal phase shifts when given either a phase delaying (CT14) or phase advancing (CT22) light pulse. B) Representative actograms from CT14 and C22 light-pulsed WT and  $CBP^{Kix/Kix}$  mice.

**Table A.1. CBP<sup>Kix/Kix</sup> gene expression experimental groups.**

<b>Group Name</b>	<b>Genotype</b>	<b>ZT time RNA isolation</b>	<b>Manipulation</b>	<b>Biological replicates (n)</b>
Wildtype home-cage	Wildtype	ZT 3.5 – ZT 4	Undisturbed	N = 4
CBP <sup>Kix/Kix</sup> home-cage	CBP <sup>Kix/Kix</sup>	ZT 3.5 – ZT 4	Undisturbed	N = 4
Wildtype fear conditioned	Wildtype	ZT 3.5 – ZT 4	Fear conditioning (ZT 3 – ZT 3.5)	N = 4
CBP <sup>Kix/Kix</sup> fear conditioned	CBP <sup>Kix/Kix</sup>	ZT 3.5 – ZT 4	Fear conditioning (ZT 3 – ZT 3.5)	N = 4



**Table A.2. Home-cage RNA-seq downregulated genes in CBP<sup>Kix/Kix</sup> mice.**

GeneID	Gene name	logFC	logCPM	LR	PValue	FDR
ENSMUSG00000032572	Col6a4	-2.598	2.733	237.135	1.66E-53	2.93E-49
ENSMUSG00000036972	Zic4	-1.181	3.254	107.659	3.19E-25	2.82E-21
ENSMUSG00000032368	Zic1	-1.218	4.838	90.978	1.45E-21	8.55E-18
ENSMUSG00000024899	Papss2	-1.041	3.930	74.218	6.99E-18	3.09E-14
ENSMUSG00000040740	Slc25a34	-1.160	1.773	64.122	1.17E-15	4.13E-12
ENSMUSG00000111971	AC158232.1	-2.074	0.349	46.999	7.10E-12	1.57E-08
ENSMUSG00000048126	Col6a3	-0.875	3.846	43.591	4.05E-11	7.94E-08
ENSMUSG00000019966	Kitl	-0.658	4.535	39.644	3.05E-10	5.38E-07
ENSMUSG00000009376	Met	-0.807	2.546	39.044	4.14E-10	6.65E-07
ENSMUSG00000068696	Gpr88	-1.777	3.071	38.371	5.85E-10	8.61E-07
ENSMUSG00000059824	Dbp	-0.526	5.216	36.382	1.62E-09	2.20E-06
ENSMUSG00000061524	Zic2	-0.608	3.912	29.086	6.92E-08	7.64E-05
ENSMUSG00000033007	Asic4	-0.550	3.071	27.463	1.60E-07	1.66E-04
ENSMUSG00000021765	Fst	-1.055	0.467	27.237	1.80E-07	1.77E-04
ENSMUSG00000068874	Selenbp1	-0.529	3.203	26.765	2.30E-07	2.13E-04
ENSMUSG00000023084	Lrrc71	-0.863	1.229	25.978	3.45E-07	2.54E-04
ENSMUSG00000038115	Ano2	-0.998	2.654	26.037	3.35E-07	2.54E-04
ENSMUSG00000021032	Ngb	-1.111	1.819	24.724	6.62E-07	4.29E-04
ENSMUSG00000002324	Rec8	-0.771	1.946	24.134	8.99E-07	5.47E-04
ENSMUSG00000049892	Rasd1	-0.564	5.150	23.924	1.00E-06	5.90E-04
ENSMUSG00000043439	Epop	-0.867	4.454	23.640	1.16E-06	6.62E-04
ENSMUSG00000040373	Cacng5	-0.620	3.741	23.323	1.37E-06	7.56E-04
ENSMUSG00000052221	Ppp1r36	-0.791	1.069	22.984	1.63E-06	8.74E-04
ENSMUSG00000027716	Trpc3	-0.506	2.993	22.851	1.75E-06	9.09E-04
ENSMUSG00000051000	Fam160a1	-0.735	3.165	22.627	1.97E-06	9.92E-04
ENSMUSG00000036578	Fxyd7	-0.421	4.982	21.866	2.92E-06	1.36E-03
ENSMUSG00000028463	Car9	-1.034	0.253	21.803	3.02E-06	1.37E-03
ENSMUSG00000087075	A230065H16Rik	-1.562	1.138	21.239	4.06E-06	1.75E-03
ENSMUSG00000039021	Ttc16	-0.681	1.411	20.743	5.25E-06	2.21E-03
ENSMUSG00000026874	Hc	-1.079	-0.135	20.345	6.47E-06	2.60E-03
ENSMUSG00000043164	Tmem212	-0.626	1.848	20.339	6.48E-06	2.60E-03
ENSMUSG00000021238	Aldh6a1	-0.343	6.174	20.204	6.96E-06	2.73E-03
ENSMUSG00000031636	Pdlim3	-0.940	0.244	20.123	7.26E-06	2.79E-03
ENSMUSG00000065922	n-R5-8s1	-3.871	3.828	20.068	7.48E-06	2.81E-03
ENSMUSG00000058135	Gstm1	-0.425	7.347	19.862	8.32E-06	3.00E-03

ENSMUSG00000029334	Prkg2	-0.582	2.210	19.491	1.01E-05	3.43E-03
ENSMUSG00000090737	Gm6788	-1.443	-0.440	19.308	1.11E-05	3.71E-03
ENSMUSG00000028339	Col15a1	-0.498	3.145	19.053	1.27E-05	4.16E-03
ENSMUSG00000029155	Spata18	-0.865	0.540	18.984	1.32E-05	4.23E-03
ENSMUSG00000106827	Gm7556	-0.937	0.319	18.924	1.36E-05	4.29E-03
ENSMUSG00000024292	Cyp4f14	-0.439	3.113	18.501	1.70E-05	5.24E-03
ENSMUSG00000038550	Ciart	-0.467	3.089	18.475	1.72E-05	5.24E-03
ENSMUSG00000102260	D330025C20Rik	-0.853	0.694	18.427	1.77E-05	5.28E-03
ENSMUSG00000084890	A830036E02Rik	-0.518	4.306	18.129	2.06E-05	5.98E-03
ENSMUSG00000032303	Chrna3	-1.434	0.045	18.096	2.10E-05	5.98E-03
ENSMUSG00000029917	C130060K24Rik	-1.280	-0.294	17.944	2.28E-05	6.09E-03
ENSMUSG00000082778	Gm15191	-1.361	-0.231	17.970	2.24E-05	6.09E-03
ENSMUSG00000084989	Crocc2	-0.663	2.428	17.967	2.25E-05	6.09E-03
ENSMUSG00000039179	Tekt5	-0.537	2.783	17.727	2.55E-05	6.72E-03
ENSMUSG00000070867	Trabd2b	-0.650	1.343	17.379	3.06E-05	7.63E-03
ENSMUSG00000083116	Gm13410	-0.836	0.462	17.375	3.07E-05	7.63E-03
ENSMUSG00000069911	Fam196b	-0.827	0.820	17.013	3.71E-05	8.86E-03
ENSMUSG00000026347	Tmem163	-0.420	3.314	16.978	3.78E-05	8.90E-03
ENSMUSG00000055653	Gpc3	-0.494	2.554	16.919	3.90E-05	9.06E-03
ENSMUSG00000035783	Acta2	-0.506	4.237	16.694	4.39E-05	1.01E-02
ENSMUSG00000021775	Nr1d2	-0.382	6.418	16.582	4.66E-05	1.02E-02
ENSMUSG00000079466	Prdm12	-1.158	-0.200	16.544	4.75E-05	1.02E-02
ENSMUSG00000092837	Rpph1	-2.723	1.423	16.638	4.52E-05	1.02E-02
ENSMUSG00000096780	Tmem181b-ps	-0.535	5.831	16.571	4.69E-05	1.02E-02
ENSMUSG00000042816	Gpr151	-1.047	1.320	16.445	5.01E-05	1.07E-02
ENSMUSG00000024027	Glp1r	-0.764	1.044	16.293	5.43E-05	1.14E-02
ENSMUSG00000009097	Tbx1	-0.922	0.804	16.233	5.60E-05	1.16E-02
ENSMUSG00000038756	Tll6	-0.699	1.189	16.175	5.77E-05	1.17E-02
ENSMUSG00000068877	Selenbp2	-0.773	0.511	16.175	5.77E-05	1.17E-02
ENSMUSG00000025784	Clec3b	-0.671	1.114	16.135	5.90E-05	1.18E-02
ENSMUSG00000037003	Tns2	-0.368	4.604	15.998	6.34E-05	1.25E-02
ENSMUSG00000064280	Ccdc146	-0.648	1.153	15.996	6.35E-05	1.25E-02
ENSMUSG00000055214	Pld5	-0.665	2.072	15.937	6.55E-05	1.27E-02
ENSMUSG00000005994	Tyrp1	-1.322	-1.042	15.847	6.87E-05	1.30E-02
ENSMUSG00000044772	Sntn	-0.931	-0.028	15.695	7.44E-05	1.37E-02
ENSMUSG00000022025	Cnmd	-1.006	1.313	15.605	7.80E-05	1.41E-02
ENSMUSG00000062372	Otof	-0.598	3.268	15.554	8.02E-05	1.42E-02
ENSMUSG00000035775	Krt20	-0.704	0.711	15.523	8.15E-05	1.43E-02

ENSMUSG00000056947	Mab21l1	-2.215	-0.621	15.450	8.47E-05	1.47E-02
ENSMUSG00000039349	C130074G19Rik	-0.427	4.447	15.129	1.00E-04	1.67E-02
ENSMUSG00000031075	Ano1	-0.494	2.887	15.027	1.06E-04	1.68E-02
ENSMUSG00000032394	Igdcc3	-0.562	1.592	15.048	1.05E-04	1.68E-02
ENSMUSG00000056972	Magel2	-0.683	2.273	14.686	1.27E-04	1.90E-02
ENSMUSG00000041703	Zic5	-0.517	2.517	14.619	1.32E-04	1.95E-02
ENSMUSG00000023064	Sneg	-0.514	2.060	14.528	1.38E-04	2.03E-02
ENSMUSG00000029177	Cenpa	-0.667	0.860	14.355	1.51E-04	2.15E-02
ENSMUSG00000041596	Nlrp5-ps	-1.001	-0.221	14.319	1.54E-04	2.16E-02
ENSMUSG00000021943	Gdf10	-0.392	3.738	14.245	1.61E-04	2.23E-02
ENSMUSG00000023267	Gabrr2	-0.836	-0.032	14.127	1.71E-04	2.36E-02
ENSMUSG00000041849	Card6	-0.487	2.124	13.948	1.88E-04	2.55E-02
ENSMUSG00000073424	Cyp4f15	-0.396	3.768	13.814	2.02E-04	2.64E-02
ENSMUSG00000026525	Opn3	-0.878	-0.108	13.792	2.04E-04	2.65E-02
ENSMUSG00000028011	Tdo2	-0.734	2.886	13.678	2.17E-04	2.80E-02
ENSMUSG00000082876	Gm11889	-0.610	1.050	13.659	2.19E-04	2.80E-02
ENSMUSG00000004328	Hif3a	-0.774	2.187	13.623	2.23E-04	2.84E-02
ENSMUSG00000023868	Pde10a	-0.322	6.294	13.594	2.27E-04	2.84E-02
ENSMUSG00000028661	Epha8	-0.738	1.949	13.576	2.29E-04	2.84E-02
ENSMUSG00000066058	Cldn19	-0.889	-0.123	13.568	2.30E-04	2.84E-02
ENSMUSG00000031789	Cngb1	-1.098	-0.376	13.545	2.33E-04	2.86E-02
ENSMUSG00000029754	Dlx6	-0.518	2.045	13.373	2.55E-04	3.09E-02
ENSMUSG00000093460	Six3os1	-0.938	0.442	13.330	2.61E-04	3.11E-02
ENSMUSG00000063704	Mapk15	-0.476	2.261	13.278	2.69E-04	3.16E-02
ENSMUSG00000090523	Gypc	-0.656	0.891	13.188	2.82E-04	3.29E-02
ENSMUSG00000018830	Myh11	-0.378	3.882	13.157	2.87E-04	3.31E-02
ENSMUSG00000024810	Il33	-0.270	5.983	13.133	2.90E-04	3.33E-02
ENSMUSG00000028971	Cort	-0.576	2.418	13.051	3.03E-04	3.43E-02
ENSMUSG00000078776	9530053A07Rik	-0.994	-0.546	13.053	3.03E-04	3.43E-02
ENSMUSG00000047139	Cd24a	-0.429	3.595	13.031	3.06E-04	3.45E-02
ENSMUSG00000022441	Efcab6	-0.415	3.311	12.944	3.21E-04	3.56E-02
ENSMUSG00000078958	Atp6ap1l	-0.788	0.643	12.950	3.20E-04	3.56E-02
ENSMUSG00000050157	Gm867	-0.617	1.071	12.896	3.29E-04	3.63E-02
ENSMUSG00000020889	Nr1d1	-0.401	6.255	12.828	3.41E-04	3.72E-02
ENSMUSG00000039634	Zfp189	-0.377	3.319	12.727	3.60E-04	3.86E-02
ENSMUSG00000048416	Mlf1	-0.650	0.890	12.740	3.58E-04	3.86E-02
ENSMUSG00000093880	Tmem181c-ps	-0.549	1.446	12.725	3.61E-04	3.86E-02
ENSMUSG00000067860	Zic3	-0.513	2.093	12.706	3.64E-04	3.86E-02

ENSMUSG00000020904	Cfap52	-0.606	1.458	12.687	3.68E-04	3.86E-02
ENSMUSG00000031927	1700012B09Rik	-0.862	-0.048	12.680	3.70E-04	3.86E-02
ENSMUSG00000021303	Gng4	-0.521	4.886	12.624	3.81E-04	3.92E-02
ENSMUSG00000030125	Lrrc23	-0.466	2.620	12.618	3.82E-04	3.92E-02
ENSMUSG00000041798	Gck	-0.689	1.434	12.341	4.43E-04	4.41E-02
ENSMUSG00000059854	Hydin	-0.572	2.122	12.335	4.45E-04	4.41E-02
ENSMUSG00000015882	Lcorl	-0.322	3.755	12.254	4.64E-04	4.58E-02
ENSMUSG00000040016	Ptger3	-0.612	1.014	12.231	4.70E-04	4.59E-02
ENSMUSG00000075020	Mir670hg	-0.478	1.968	12.225	4.71E-04	4.59E-02
ENSMUSG00000044072	Eml6	-0.299	5.080	12.173	4.85E-04	4.68E-02
ENSMUSG00000070306	Ccdc153	-0.465	2.694	12.149	4.91E-04	4.71E-02
ENSMUSG00000041741	Pde3a	-0.433	2.555	12.133	4.95E-04	4.73E-02
ENSMUSG00000030109	Slc6a12	-0.669	0.911	12.096	5.05E-04	4.79E-02
ENSMUSG00000047686	Zcchc5	-1.421	2.107	12.089	5.07E-04	4.79E-02
ENSMUSG00000022262	Dnah5	-0.387	2.585	12.022	5.26E-04	4.94E-02
ENSMUSG00000051910	Sox6	-0.298	4.107	12.005	5.31E-04	4.96E-02

**Table A.3. Home-cage RNA-seq upregulated genes in CBP<sup>Kix/Kix</sup> mice.**

GeneID	Gene name	logFC	logCPM	LR	PValue	FDR
ENSMUSG00000003545	Fosb	0.937	4.135	55.313	1.03E-13	3.03E-10
ENSMUSG000000073418	C4b	0.551	6.449	51.465	7.29E-13	1.84E-09
ENSMUSG000000060735	Rxfp3	0.574	3.191	33.891	5.83E-09	7.35E-06
ENSMUSG000000020846	Rflnb	0.495	4.145	30.876	2.75E-08	3.24E-05
ENSMUSG000000028076	Cd1d1	0.897	0.876	26.438	2.72E-07	2.40E-04
ENSMUSG000000029032	Arhgef16	1.151	-0.585	26.020	3.38E-07	2.54E-04
ENSMUSG000000032128	Robo3	0.447	6.388	26.165	3.13E-07	2.54E-04
ENSMUSG000000026043	Col3a1	0.922	2.783	25.152	5.30E-07	3.74E-04
ENSMUSG000000020932	Gfap	0.504	9.378	24.672	6.80E-07	4.29E-04
ENSMUSG000000045573	Penk	1.409	6.287	24.766	6.47E-07	4.29E-04
ENSMUSG000000038173	Enpp6	0.637	3.359	22.519	2.08E-06	1.02E-03
ENSMUSG000000037071	Scd1	0.367	7.828	22.172	2.49E-06	1.19E-03
ENSMUSG000000026205	Slc23a3	1.206	-0.622	21.394	3.74E-06	1.65E-03
ENSMUSG000000023918	Adgrf4	0.545	2.232	19.977	7.84E-06	2.88E-03
ENSMUSG000000037868	Egr2	1.075	1.955	19.797	8.61E-06	3.04E-03
ENSMUSG000000032500	Dclk3	0.392	4.530	19.565	9.72E-06	3.37E-03
ENSMUSG000000085235	Gm12576	0.962	-0.569	18.276	1.91E-05	5.62E-03
ENSMUSG000000034855	Cxcl10	1.165	-1.030	18.014	2.19E-05	6.09E-03
ENSMUSG000000030713	Klk7	0.954	-0.434	17.688	2.60E-05	6.76E-03
ENSMUSG000000025572	Tmc6	0.831	3.791	17.635	2.68E-05	6.85E-03
ENSMUSG000000056749	Nfil3	0.393	3.497	17.347	3.11E-05	7.64E-03
ENSMUSG000000054944	5330416C01Rik	1.009	-0.364	17.293	3.20E-05	7.75E-03
ENSMUSG000000053560	Ier2	0.499	2.531	16.560	4.71E-05	1.02E-02
ENSMUSG000000048191	Muc6	0.672	0.676	15.859	6.83E-05	1.30E-02
ENSMUSG000000046718	Bst2	0.494	2.024	15.795	7.06E-05	1.33E-02
ENSMUSG000000013766	Ly6g6e	0.537	1.792	15.741	7.26E-05	1.35E-02
ENSMUSG000000061808	Ttr	2.633	9.557	15.671	7.54E-05	1.37E-02
ENSMUSG000000099440	Gm29593	1.244	-1.585	15.572	7.94E-05	1.42E-02
ENSMUSG000000027315	Spint1	0.533	1.638	15.412	8.64E-05	1.48E-02
ENSMUSG000000032487	Ptgs2	0.790	6.061	15.255	9.39E-05	1.59E-02
ENSMUSG000000050201	Otop2	0.999	-0.730	15.214	9.60E-05	1.61E-02
ENSMUSG000000000730	Dnmt3l	1.032	-0.960	15.113	1.01E-04	1.67E-02
ENSMUSG000000039405	Prss23	0.534	4.110	15.090	1.03E-04	1.68E-02
ENSMUSG000000000216	Scnn1g	1.201	-1.431	14.987	1.08E-04	1.68E-02
ENSMUSG000000046207	Pik3r6	0.534	1.752	15.010	1.07E-04	1.68E-02

ENSMUSG00000055415	Atp10b	0.544	1.467	14.991	1.08E-04	1.68E-02
ENSMUSG00000061132	Blnk	0.471	2.698	15.018	1.06E-04	1.68E-02
ENSMUSG00000024907	Gal	0.952	-0.197	14.845	1.17E-04	1.79E-02
ENSMUSG00000005958	Ephb3	0.328	4.608	14.815	1.19E-04	1.80E-02
ENSMUSG00000022324	Matn2	0.268	6.563	14.718	1.25E-04	1.88E-02
ENSMUSG00000072720	Myo18b	0.505	1.783	14.495	1.41E-04	2.05E-02
ENSMUSG00000001025	S100a6	0.377	3.464	14.429	1.46E-04	2.11E-02
ENSMUSG00000026312	Cdh7	0.448	3.072	14.411	1.47E-04	2.11E-02
ENSMUSG00000063632	Sox11	0.375	4.377	14.340	1.53E-04	2.15E-02
ENSMUSG00000031557	Plekha2	0.448	5.612	13.992	1.84E-04	2.51E-02
ENSMUSG00000075334	Rprm	0.762	5.526	13.932	1.90E-04	2.55E-02
ENSMUSG00000032332	Col12a1	0.473	3.520	13.883	1.95E-04	2.60E-02
ENSMUSG00000037686	Aspg	0.631	2.534	13.869	1.96E-04	2.60E-02
ENSMUSG00000054966	Lmntd1	0.799	-0.294	13.843	1.99E-04	2.62E-02
ENSMUSG00000057440	Mpp7	0.480	2.163	13.597	2.26E-04	2.84E-02
ENSMUSG00000028602	Tnfrsf8	0.612	0.958	13.488	2.40E-04	2.92E-02
ENSMUSG00000007080	Pole	0.524	1.454	13.346	2.59E-04	3.11E-02
ENSMUSG00000019960	Dusp6	0.316	6.000	13.318	2.63E-04	3.11E-02
ENSMUSG00000030270	Cpne9	0.462	4.751	13.174	2.84E-04	3.30E-02
ENSMUSG00000056596	Trnp1	0.341	7.270	12.885	3.31E-04	3.63E-02
ENSMUSG00000009214	Tmem8c	0.931	-1.063	12.703	3.65E-04	3.86E-02
ENSMUSG00000036907	C1ql2	0.367	6.797	12.658	3.74E-04	3.88E-02
ENSMUSG00000026630	Batf3	0.783	-0.034	12.478	4.12E-04	4.20E-02
ENSMUSG00000020847	Rph3al	0.384	2.504	12.445	4.19E-04	4.25E-02
ENSMUSG00000028749	Pla2g2f	0.480	2.036	12.435	4.21E-04	4.25E-02
ENSMUSG00000031170	Slc38a5	0.888	2.716	12.417	4.26E-04	4.27E-02
ENSMUSG00000041695	Kcnj2	0.494	4.347	12.219	4.73E-04	4.59E-02

**Table A.4. Fear conditioned RNA-seq downregulated genes in CBP<sup>Kix/Kix</sup> mice.**

GeneID	Gene name	logFC	logCPM	LR	PValue	FDR
ENSMUSG00000048108	Tmem72	-2.425	3.771	327.830	2.85E-73	4.90E-69
ENSMUSG00000024899	Papss2	-1.187	3.807	300.888	2.11E-67	1.81E-63
ENSMUSG00000004655	Aqp1	-1.604	3.665	194.417	3.45E-44	1.98E-40
ENSMUSG00000079436	Kcnj13	-2.168	2.597	144.245	3.14E-33	1.35E-29
ENSMUSG00000032572	Col6a4	-1.422	2.780	134.162	5.04E-31	1.73E-27
ENSMUSG00000059824	Dbp	-0.481	5.463	113.149	2.00E-26	5.73E-23
ENSMUSG00000059991	Nptx2	-0.589	4.845	100.171	1.40E-23	3.43E-20
ENSMUSG00000037086	Prr32	-2.069	1.919	95.558	1.44E-22	3.08E-19
ENSMUSG00000021250	Fos	-0.511	4.872	76.964	1.74E-18	3.32E-15
ENSMUSG00000052837	Junb	-0.349	6.315	73.032	1.28E-17	2.19E-14
ENSMUSG00000015652	Steap1	-1.391	1.885	63.544	1.57E-15	2.34E-12
ENSMUSG00000040740	Slc25a34	-1.086	1.591	63.459	1.64E-15	2.34E-12
ENSMUSG00000055235	Wdr86	-1.186	2.404	62.556	2.59E-15	3.42E-12
ENSMUSG00000014158	Trpv4	-0.755	3.220	61.008	5.68E-15	6.51E-12
ENSMUSG00000043439	Epop	-0.436	4.828	61.056	5.55E-15	6.51E-12
ENSMUSG00000016024	Lbp	-0.665	3.914	58.536	2.00E-14	2.02E-11
ENSMUSG00000056174	Col8a2	-0.650	3.486	58.641	1.89E-14	2.02E-11
ENSMUSG00000028125	Abca4	-0.625	3.863	58.161	2.42E-14	2.31E-11
ENSMUSG00000020893	Per1	-0.288	6.775	55.374	9.97E-14	9.01E-11
ENSMUSG00000027489	Necab3	-0.528	4.296	55.198	1.09E-13	9.37E-11
ENSMUSG00000023034	Nr4a1	-0.283	6.726	54.806	1.33E-13	1.09E-10
ENSMUSG00000058135	Gstm1	-0.344	7.380	54.665	1.43E-13	1.12E-10
ENSMUSG00000021680	Crhbp	-0.453	5.001	53.190	3.03E-13	2.14E-10
ENSMUSG00000024810	Il33	-0.313	6.045	51.794	6.16E-13	4.07E-10
ENSMUSG00000038550	Ciart	-0.548	3.424	51.327	7.82E-13	4.98E-10
ENSMUSG00000020889	Nr1d1	-0.295	6.397	49.566	1.92E-12	1.14E-09
ENSMUSG00000111971	AC158232.1	-1.970	-0.123	49.336	2.16E-12	1.24E-09
ENSMUSG00000068323	Slc4a5	-1.613	3.873	47.976	4.31E-12	2.39E-09
ENSMUSG00000029135	Fosl2	-0.348	5.924	47.814	4.69E-12	2.52E-09
ENSMUSG00000026051	1500015O10Rik	-0.877	4.182	47.712	4.94E-12	2.57E-09
ENSMUSG00000072845	Tmprss11a	-2.098	0.724	46.545	8.95E-12	4.39E-09
ENSMUSG00000047230	Cldn2	-1.085	3.068	44.512	2.53E-11	1.21E-08
ENSMUSG00000001827	Folr1	-1.071	3.822	42.362	7.59E-11	3.52E-08
ENSMUSG00000039179	Tekt5	-0.602	2.690	41.912	9.55E-11	4.32E-08
ENSMUSG00000017493	Igfbp4	-0.255	6.129	41.573	1.14E-10	5.00E-08

ENSMUSG00000050121	Opalin	-0.331	4.817	41.270	1.33E-10	5.56E-08
ENSMUSG00000057880	Abat	-0.183	8.073	41.279	1.32E-10	5.56E-08
ENSMUSG00000020681	Ace	-0.598	5.472	40.806	1.68E-10	6.88E-08
ENSMUSG00000029055	Plch2	-0.201	7.550	40.585	1.88E-10	7.52E-08
ENSMUSG00000012819	Cdh23	-0.906	1.471	39.632	3.07E-10	1.17E-07
ENSMUSG00000024190	Dusp1	-0.427	4.624	38.692	4.96E-10	1.85E-07
ENSMUSG000000107369	Gstm2-ps1	-0.434	3.705	38.636	5.11E-10	1.87E-07
ENSMUSG00000021238	Aldh6a1	-0.229	6.233	37.506	9.11E-10	3.26E-07
ENSMUSG00000030450	Oca2	-1.281	1.200	37.405	9.60E-10	3.37E-07
ENSMUSG00000017723	Wfdc2	-1.276	1.262	36.743	1.35E-09	4.63E-07
ENSMUSG00000004366	Sst	-0.228	7.569	36.689	1.39E-09	4.67E-07
ENSMUSG00000015312	Gadd45b	-0.396	3.828	35.807	2.18E-09	7.06E-07
ENSMUSG00000010080	Epn3	-0.629	2.677	35.233	2.93E-09	9.31E-07
ENSMUSG00000037490	Slc2a12	-0.514	3.656	34.846	3.57E-09	1.10E-06
ENSMUSG00000039672	Kcne2	-1.314	3.390	33.414	7.45E-09	2.21E-06
ENSMUSG00000022237	Ankrd33b	-0.233	6.440	32.734	1.06E-08	3.03E-06
ENSMUSG00000028957	Per3	-0.262	5.831	32.739	1.05E-08	3.03E-06
ENSMUSG00000024039	Cbs	-0.266	5.164	32.642	1.11E-08	3.12E-06
ENSMUSG00000020423	Btg2	-0.370	5.019	32.448	1.22E-08	3.39E-06
ENSMUSG00000022505	Emp2	-0.401	4.519	32.205	1.39E-08	3.78E-06
ENSMUSG00000026826	Nr4a2	-0.367	6.262	32.153	1.42E-08	3.82E-06
ENSMUSG00000044177	Wfikkn2	-0.991	1.860	31.868	1.65E-08	4.36E-06
ENSMUSG00000032515	Csrnp1	-0.415	3.530	31.036	2.53E-08	6.50E-06
ENSMUSG00000039457	Ppl	-0.504	2.800	30.863	2.77E-08	7.00E-06
ENSMUSG00000013523	Bcas1	-0.218	7.020	29.544	5.47E-08	1.32E-05
ENSMUSG00000055866	Per2	-0.304	5.605	29.227	6.44E-08	1.52E-05
ENSMUSG00000026579	F5	-1.209	4.431	28.553	9.12E-08	2.06E-05
ENSMUSG00000021587	Pcsk1	-0.366	3.796	28.197	1.10E-07	2.45E-05
ENSMUSG00000030088	Aldh1l1	-0.252	5.580	27.796	1.35E-07	2.93E-05
ENSMUSG00000057969	Sema3b	-0.529	3.135	27.746	1.38E-07	2.97E-05
ENSMUSG00000032355	Mlip	-0.285	4.880	27.588	1.50E-07	3.18E-05
ENSMUSG00000038375	Trp53inp2	-0.192	7.478	27.522	1.55E-07	3.25E-05
ENSMUSG00000032246	Calml4	-0.592	2.613	27.248	1.79E-07	3.70E-05
ENSMUSG00000022197	Pdzd2	-0.309	5.840	26.829	2.22E-07	4.55E-05
ENSMUSG00000024292	Cyp4f14	-0.444	3.013	26.716	2.36E-07	4.77E-05
ENSMUSG00000022512	Cldn1	-0.802	2.409	26.682	2.40E-07	4.79E-05
ENSMUSG00000003477	Inmt	-0.914	1.002	26.614	2.48E-07	4.81E-05
ENSMUSG00000020932	Gfap	-0.233	8.460	26.631	2.46E-07	4.81E-05



ENSMUSG00000052632	Asap2	-0.208	5.813	26.609	2.49E-07	4.81E-05
ENSMUSG00000005268	Prir	-0.486	3.925	26.490	2.65E-07	5.06E-05
ENSMUSG00000091415	Ak9	-0.886	0.775	26.312	2.90E-07	5.48E-05
ENSMUSG00000027858	Tspan2	-0.234	7.008	26.161	3.14E-07	5.83E-05
ENSMUSG000000109372	Gm19410	-0.324	4.261	26.154	3.15E-07	5.83E-05
ENSMUSG00000031786	Drc7	-0.423	3.733	25.811	3.77E-07	6.88E-05
ENSMUSG00000015002	Efr3a	-0.196	7.482	25.773	3.84E-07	6.94E-05
ENSMUSG00000029822	Osbpl3	-0.308	4.783	25.535	4.34E-07	7.77E-05
ENSMUSG00000037428	Vgf	-0.223	7.252	25.154	5.29E-07	9.38E-05
ENSMUSG00000028328	Tmod1	-0.269	4.729	24.930	5.94E-07	0.000104
ENSMUSG00000037868	Egr2	-0.458	2.733	24.638	6.92E-07	0.00012
ENSMUSG00000025212	Sfxn3	-0.164	6.734	24.578	7.14E-07	0.000123
ENSMUSG00000043671	Dpy19l3	-0.225	5.906	24.555	7.22E-07	0.000123
ENSMUSG00000022090	Pdlim2	-0.376	3.440	24.528	7.32E-07	0.000123
ENSMUSG00000023043	Krt18	-0.786	1.956	24.468	7.55E-07	0.000126
ENSMUSG00000027570	Col9a3	-0.395	5.504	24.133	8.99E-07	0.000149
ENSMUSG00000022054	Nefm	-0.169	7.299	23.965	9.81E-07	0.000161
ENSMUSG00000023019	Gpd1	-0.166	6.886	23.773	1.08E-06	0.000175
ENSMUSG00000066129	Kndc1	-0.150	7.612	23.749	1.10E-06	0.000175
ENSMUSG00000031883	Car7	-0.309	4.647	23.717	1.12E-06	0.000176
ENSMUSG00000041642	Kif21b	-0.210	6.971	22.972	1.64E-06	0.00025
ENSMUSG00000021091	Serpina3n	-0.269	5.707	22.776	1.82E-06	0.00027
ENSMUSG00000068874	Selenbp1	-0.390	3.233	22.773	1.82E-06	0.00027
ENSMUSG00000085609	1700016P03Rik	-0.432	2.697	22.612	1.98E-06	0.000291
ENSMUSG00000016494	Cd34	-0.207	5.609	22.594	2.00E-06	0.000291
ENSMUSG00000020486	Sept4	-0.186	7.614	22.541	2.06E-06	0.000297
ENSMUSG00000025911	Adhfe1	-0.286	4.283	22.363	2.26E-06	0.000323
ENSMUSG00000062591	Tubb4a	-0.119	9.437	22.337	2.29E-06	0.000325
ENSMUSG00000020473	Aebp1	-0.314	3.941	22.148	2.52E-06	0.000351
ENSMUSG00000022797	Tfrc	-0.172	6.982	22.143	2.53E-06	0.000351
ENSMUSG00000061048	Cdh3	-1.493	0.424	21.801	3.02E-06	0.000416
ENSMUSG00000017868	Sgk2	-0.819	0.875	21.777	3.06E-06	0.000418
ENSMUSG00000026841	Fibcd1	-0.226	7.392	21.730	3.14E-06	0.000423
ENSMUSG00000034739	Mfrp	-1.410	3.963	21.706	3.18E-06	0.000423
ENSMUSG00000054986	Sec14l3	-0.718	1.084	21.713	3.17E-06	0.000423
ENSMUSG00000022441	Efcab6	-0.390	3.145	21.571	3.41E-06	0.000451
ENSMUSG00000041708	Mpped1	-0.243	7.770	21.540	3.47E-06	0.000455
ENSMUSG00000001467	Cyp51	-0.188	5.975	21.511	3.52E-06	0.000458

ENSMUSG00000042608	Stk40	-0.225	5.183	21.495	3.55E-06	0.000458
ENSMUSG00000059898	Dsc3	-0.659	1.396	21.422	3.69E-06	0.000473
ENSMUSG00000022032	Scara5	-0.954	0.824	21.357	3.81E-06	0.000485
ENSMUSG00000022893	Adamts1	-0.262	4.926	21.214	4.11E-06	0.000511
ENSMUSG00000028179	Cth	-0.595	1.664	20.993	4.61E-06	0.000569
ENSMUSG00000030342	Cd9	-0.236	5.299	20.983	4.63E-06	0.000569
ENSMUSG00000033595	Lgi3	-0.162	7.231	20.939	4.74E-06	0.000574
ENSMUSG00000045294	Insig1	-0.259	6.197	20.877	4.90E-06	0.000584
ENSMUSG00000026204	Ptpn	-0.132	9.159	20.854	4.96E-06	0.000587
ENSMUSG00000064246	Chil1	-0.389	2.951	20.701	5.37E-06	0.000632
ENSMUSG00000021182	Ccdc88c	-0.189	5.651	20.250	6.80E-06	0.000789
ENSMUSG00000108126	Gm43909	-0.767	0.830	19.964	7.89E-06	0.000904
ENSMUSG00000017400	Stac2	-0.210	6.412	19.950	7.95E-06	0.000905
ENSMUSG00000063558	Aox1	-0.342	3.508	19.834	8.45E-06	0.000955
ENSMUSG00000048038	Ccdc187	-0.354	3.258	19.630	9.40E-06	0.001056
ENSMUSG00000015806	Qdpr	-0.148	7.115	19.558	9.76E-06	0.001089
ENSMUSG00000063434	Sorcs3	-0.225	5.791	19.452	1.03E-05	0.001143
ENSMUSG00000004105	Angptl2	-0.466	2.535	19.172	1.19E-05	0.001283
ENSMUSG00000021848	Otx2	-0.630	3.136	19.185	1.19E-05	0.001283
ENSMUSG00000023805	Synj2	-0.154	6.553	19.182	1.19E-05	0.001283
ENSMUSG00000038457	Tmem255b	-0.640	1.357	19.086	1.25E-05	0.001333
ENSMUSG00000040836	Gpr161	-0.222	6.300	19.071	1.26E-05	0.001336
ENSMUSG00000019970	Sgk1	-0.189	6.343	19.035	1.28E-05	0.001353
ENSMUSG00000042489	Clspn	-0.685	1.077	18.955	1.34E-05	0.001385
ENSMUSG00000067879	3110035E14Rik	-0.233	7.667	18.932	1.35E-05	0.001394
ENSMUSG00000025855	Prkar1b	-0.103	9.193	18.900	1.38E-05	0.001409
ENSMUSG00000003518	Dusp3	-0.142	6.856	18.603	1.61E-05	0.001617
ENSMUSG00000039202	Abhd2	-0.160	6.850	18.525	1.68E-05	0.001675
ENSMUSG00000031351	Zfp185	-0.388	2.880	18.464	1.73E-05	0.00172
ENSMUSG00000027894	Slc6a17	-0.112	9.118	18.353	1.84E-05	0.001812
ENSMUSG00000020604	Arsg	-0.210	5.058	18.256	1.93E-05	0.001896
ENSMUSG00000039633	Lonrf1	-0.208	5.098	18.205	1.98E-05	0.001925
ENSMUSG00000031425	Plp1	-0.126	10.695	18.159	2.03E-05	0.001962
ENSMUSG00000026418	Tnni1	-0.479	2.101	18.142	2.05E-05	0.001968
ENSMUSG00000079056	Kcnip3	-0.215	6.610	18.123	2.07E-05	0.001976
ENSMUSG00000070780	Rbm47	-0.780	1.267	18.045	2.16E-05	0.002048
ENSMUSG00000042589	Cux2	-0.238	4.993	17.968	2.25E-05	0.002109
ENSMUSG00000025981	Coq10b	-0.298	4.172	17.810	2.44E-05	0.002267

ENSMUSG00000027520	Zdbf2	-0.272	3.982	17.794	2.46E-05	0.002274
ENSMUSG0000001435	Col18a1	-0.346	3.182	17.727	2.55E-05	0.002343
ENSMUSG00000044229	Nxpe4	-0.278	4.141	17.696	2.59E-05	0.002369
ENSMUSG00000051067	Lingo3	-0.225	5.477	17.656	2.65E-05	0.002407
ENSMUSG00000030123	Plxnd1	-0.203	5.336	17.535	2.82E-05	0.002498
ENSMUSG00000033316	Galnt9	-0.165	6.764	17.547	2.80E-05	0.002498
ENSMUSG00000054889	Dsp	-0.188	5.613	17.538	2.82E-05	0.002498
ENSMUSG00000036169	Sostdc1	-0.767	3.839	17.518	2.85E-05	0.002508
ENSMUSG00000024597	Slc12a2	-0.187	6.361	17.374	3.07E-05	0.002664
ENSMUSG00000065922	n-R5-8s1	-2.246	4.550	17.379	3.06E-05	0.002664
ENSMUSG00000037992	Rara	-0.297	3.922	17.354	3.10E-05	0.002666
ENSMUSG00000042453	Reln	-0.179	6.111	17.357	3.10E-05	0.002666
ENSMUSG00000027375	Mal	-0.158	7.749	17.268	3.25E-05	0.002775
ENSMUSG00000024558	Mapk4	-0.195	5.839	17.248	3.28E-05	0.002791
ENSMUSG00000029819	Npy	-0.190	5.335	17.076	3.59E-05	0.003011
ENSMUSG00000034825	Nrip3	-0.170	7.691	16.941	3.86E-05	0.003206
ENSMUSG00000049649	Gpr3	-0.417	2.459	16.938	3.86E-05	0.003206
ENSMUSG00000014496	Ankrd28	-0.174	5.687	16.925	3.89E-05	0.003208
ENSMUSG00000068566	Myadm	-0.172	5.963	16.918	3.90E-05	0.003208
ENSMUSG00000031266	Gla	-0.351	3.186	16.831	4.09E-05	0.003339
ENSMUSG00000027210	Meis2	-0.210	5.810	16.805	4.14E-05	0.003357
ENSMUSG00000110935	AC138292.1	-0.571	1.431	16.788	4.18E-05	0.003371
ENSMUSG00000013089	Etv5	-0.174	6.043	16.738	4.29E-05	0.003446
ENSMUSG00000051920	Rspo2	-0.360	4.847	16.673	4.44E-05	0.003534
ENSMUSG00000011148	Adssl1	-0.266	3.957	16.654	4.49E-05	0.003553
ENSMUSG00000044976	Wdr72	-1.297	-0.058	16.528	4.79E-05	0.003745
ENSMUSG00000014602	Kif1a	-0.098	10.416	16.493	4.88E-05	0.003779
ENSMUSG00000035686	Thrsp	-0.219	4.751	16.414	5.09E-05	0.003904
ENSMUSG00000032280	Tle3	-0.178	5.506	16.402	5.12E-05	0.003913
ENSMUSG00000076439	Mog	-0.169	6.033	16.120	5.94E-05	0.0045
ENSMUSG00000022425	Enpp2	-0.491	9.983	16.029	6.24E-05	0.004702
ENSMUSG00000028876	Epha10	-0.183	5.982	15.959	6.47E-05	0.004819
ENSMUSG00000104093	A330015K06Rik	-0.706	0.670	15.969	6.44E-05	0.004819
ENSMUSG00000040435	Ppp1r15a	-0.249	4.080	15.781	7.11E-05	0.005245
ENSMUSG00000033952	Aspm	-1.261	-0.773	15.766	7.17E-05	0.005265
ENSMUSG00000061808	Ttr	-1.253	10.938	15.564	7.98E-05	0.005833
ENSMUSG00000051985	Igfn1	-0.756	2.134	15.537	8.09E-05	0.00589
ENSMUSG00000036634	Mag	-0.119	7.680	15.509	8.21E-05	0.005954

ENSMUSG00000073424	Cyp4f15	-0.264	3.779	15.485	8.31E-05	0.006003
ENSMUSG00000100706	Gm19744	-0.544	1.443	15.463	8.41E-05	0.006048
ENSMUSG00000028962	Slc4a2	-0.168	5.695	15.452	8.46E-05	0.006059
ENSMUSG00000003545	Fosb	-0.326	3.881	15.346	8.95E-05	0.006357
ENSMUSG00000016763	Scube1	-0.189	5.047	15.297	9.19E-05	0.00647
ENSMUSG00000068196	Col8a1	-0.916	3.268	15.301	9.17E-05	0.00647
ENSMUSG00000029455	Aldh2	-0.176	6.395	15.209	9.63E-05	0.006724
ENSMUSG00000026688	Mgst3	-0.214	5.342	15.200	9.67E-05	0.006728
ENSMUSG00000027347	Rasgrp1	-0.106	9.414	15.108	0.000102	0.007007
ENSMUSG00000038910	Plcl2	-0.164	5.980	15.112	0.000101	0.007007
ENSMUSG00000029467	Atp2a2	-0.088	9.881	14.984	0.000108	0.007392
ENSMUSG00000021775	Nr1d2	-0.218	6.585	14.960	0.00011	0.007429
ENSMUSG00000047842	Diras2	-0.129	8.411	14.953	0.00011	0.007429
ENSMUSG00000049382	Krt8	-0.714	1.055	14.956	0.00011	0.007429
ENSMUSG00000000184	Ccnd2	-0.145	6.544	14.937	0.000111	0.007454
ENSMUSG00000003849	Nqo1	-0.427	2.272	14.888	0.000114	0.007562
ENSMUSG00000030905	Crym	-0.119	7.990	14.882	0.000114	0.007562
ENSMUSG00000091636	Akain1	-0.252	4.080	14.783	0.000121	0.00788
ENSMUSG00000024042	Sik1	-0.226	4.282	14.739	0.000123	0.008005
ENSMUSG00000092090	Gm3294	-0.448	2.019	14.693	0.000126	0.00817
ENSMUSG00000055078	Gabra5	-0.120	7.876	14.627	0.000131	0.008402
ENSMUSG00000015354	Pcolce2	-0.307	3.325	14.607	0.000132	0.008458
ENSMUSG00000053025	Sv2b	-0.113	9.042	14.511	0.000139	0.008834
ENSMUSG00000008855	Hdac5	-0.135	7.517	14.480	0.000142	0.008948
ENSMUSG00000108434	Gm45768	-0.350	2.675	14.359	0.000151	0.009507
ENSMUSG00000003526	Prodh	-0.238	5.006	14.283	0.000157	0.009864
ENSMUSG00000011154	Cfap161	-0.566	1.139	14.239	0.000161	0.010062
ENSMUSG00000034765	Dusp5	-0.226	4.774	14.208	0.000164	0.010191
ENSMUSG00000067578	Cbln4	-0.281	3.996	14.157	0.000168	0.01043
ENSMUSG00000028348	Murc	-0.754	0.355	14.149	0.000169	0.010437
ENSMUSG00000040093	Bmf	-0.447	2.049	14.041	0.000179	0.011015
ENSMUSG00000023267	Gabbr2	-0.838	0.212	13.856	0.000197	0.012071
ENSMUSG00000016624	Phf21b	-0.287	3.551	13.827	0.0002	0.012211
ENSMUSG00000004637	Wwox	-0.228	4.117	13.650	0.00022	0.013144
ENSMUSG00000028864	Hgf	-0.538	1.229	13.665	0.000219	0.013144
ENSMUSG00000053644	Aldh7a1	-0.138	6.527	13.652	0.00022	0.013144
ENSMUSG00000032232	Cgnl1	-0.224	4.815	13.612	0.000225	0.013335
ENSMUSG00000050558	Prokr2	-0.394	2.284	13.609	0.000225	0.013335

ENSMUSG00000041390	Mdfic	-0.383	2.544	13.562	0.000231	0.013583
ENSMUSG00000053646	Plxnb1	-0.129	7.058	13.566	0.00023	0.013583
ENSMUSG00000068220	Lgals1	-0.283	3.398	13.509	0.000237	0.013828
ENSMUSG00000027171	Prrg4	-0.987	-0.336	13.435	0.000247	0.014221
ENSMUSG00000028919	Arhgef19	-0.199	4.565	13.431	0.000247	0.014221
ENSMUSG00000030889	Vwa3a	-0.218	4.432	13.438	0.000247	0.014221
ENSMUSG00000042734	Ttc9	-0.170	5.540	13.359	0.000257	0.014678
ENSMUSG00000053846	Lipg	-0.477	1.603	13.338	0.00026	0.0148
ENSMUSG00000016179	Camk1g	-0.179	5.187	13.217	0.000277	0.01563
ENSMUSG00000027004	Frzb	-0.198	4.931	13.192	0.000281	0.015787
ENSMUSG00000020953	Coch	-0.308	3.213	13.165	0.000285	0.015869
ENSMUSG00000033730	Egr3	-0.198	5.482	13.174	0.000284	0.015869
ENSMUSG00000046402	Rbp1	-0.258	3.747	13.164	0.000285	0.015869
ENSMUSG00000022055	Nefl	-0.108	8.369	13.116	0.000293	0.016226
ENSMUSG00000039115	Itga9	-0.370	2.355	13.038	0.000305	0.016746
ENSMUSG00000056966	Gjc3	-0.232	4.300	13.033	0.000306	0.016746
ENSMUSG00000022949	Clic6	-0.841	5.008	13.026	0.000307	0.01676
ENSMUSG00000039741	Bahcc1	-0.196	5.242	12.906	0.000328	0.017758
ENSMUSG00000026141	Col19a1	-0.319	2.762	12.886	0.000331	0.017888
ENSMUSG00000032988	Slc16a8	-0.541	1.713	12.724	0.000361	0.019383
ENSMUSG00000049907	Rasl11b	-0.147	5.990	12.687	0.000368	0.019708
ENSMUSG00000036304	Zdhhc23	-0.243	3.830	12.584	0.000389	0.020507
ENSMUSG00000028698	Pik3r3	-0.167	5.605	12.476	0.000412	0.021572
ENSMUSG00000019890	Nts	-0.331	2.793	12.453	0.000417	0.021725
ENSMUSG00000026697	Myoc	-0.230	4.027	12.446	0.000419	0.021751
ENSMUSG00000010660	Plcd1	-0.244	3.884	12.419	0.000425	0.02186
ENSMUSG00000026836	Acvr1	-0.157	5.438	12.421	0.000425	0.02186
ENSMUSG00000022449	Adams20	-0.193	4.532	12.411	0.000427	0.021888
ENSMUSG00000008153	Clstn3	-0.096	8.586	12.331	0.000445	0.022713
ENSMUSG00000021670	Hmgcr	-0.149	6.965	12.306	0.000452	0.022889
ENSMUSG00000033007	Asic4	-0.329	3.037	12.274	0.000459	0.023208
ENSMUSG00000097789	Gm2115	-0.188	6.747	12.207	0.000476	0.023919
ENSMUSG00000048537	Phldb1	-0.125	7.445	12.180	0.000483	0.024193
ENSMUSG00000097023	Mir9-3hg	-0.198	4.946	12.167	0.000486	0.0243
ENSMUSG00000029093	Sorcs2	-0.131	6.251	12.100	0.000504	0.024896
ENSMUSG00000019194	Scn1b	-0.093	8.602	12.073	0.000511	0.025087
ENSMUSG00000027115	Kif18a	-0.674	0.405	12.066	0.000514	0.025087
ENSMUSG00000027332	lvd	-0.125	6.880	12.074	0.000511	0.025087

ENSMUSG00000046805	Mpeg1	-0.190	4.659	12.064	0.000514	0.025087
ENSMUSG00000052221	Ppp1r36	-0.490	1.344	11.988	0.000535	0.025915
ENSMUSG00000023232	Serinc2	-0.268	3.391	11.945	0.000548	0.026454
ENSMUSG00000027340	Slc23a2	-0.123	7.531	11.869	0.000571	0.027378
ENSMUSG00000059013	Sh2d3c	-0.214	4.443	11.865	0.000572	0.027378
ENSMUSG00000038264	Sema7a	-0.105	7.264	11.838	0.00058	0.027626
ENSMUSG00000059049	Frem1	-0.307	3.136	11.803	0.000592	0.027949
ENSMUSG00000068099	1500009C09Rik	-0.130	6.170	11.805	0.000591	0.027949
ENSMUSG00000026028	Trak2	-0.095	7.888	11.784	0.000597	0.027981
ENSMUSG00000030102	Itpr1	-0.131	8.356	11.789	0.000596	0.027981
ENSMUSG00000038010	Ccdc138	-0.364	2.252	11.783	0.000598	0.027981
ENSMUSG00000026939	Tmem141	-0.245	3.579	11.757	0.000606	0.028309
ENSMUSG00000025380	Fscn2	-0.904	-0.456	11.699	0.000625	0.028582
ENSMUSG00000030220	Arhgdib	-0.328	2.614	11.708	0.000622	0.028582
ENSMUSG00000038530	Rgs4	-0.212	7.479	11.699	0.000625	0.028582
ENSMUSG00000038665	Dgki	-0.268	3.476	11.718	0.000619	0.028582
ENSMUSG00000042745	Id1	-0.268	3.378	11.716	0.00062	0.028582
ENSMUSG00000059248	Sept9	-0.145	6.801	11.725	0.000617	0.028582
ENSMUSG00000059921	Unc5c	-0.155	5.660	11.713	0.000621	0.028582
ENSMUSG00000000253	Gmpr	-0.149	5.951	11.656	0.00064	0.029171
ENSMUSG000000109572	Cfap99	-0.806	-0.105	11.621	0.000652	0.029569
ENSMUSG00000036591	Arhgap21	-0.096	8.200	11.540	0.000681	0.030795
ENSMUSG000000103770	Pcdha9	-0.347	2.394	11.493	0.000699	0.031259
ENSMUSG00000018566	Slc2a4	-0.821	0.003	11.440	0.000719	0.031839
ENSMUSG00000022026	Olfm4	-0.360	2.374	11.437	0.00072	0.031839
ENSMUSG00000025558	Dock9	-0.086	8.246	11.454	0.000713	0.031839
ENSMUSG00000035226	Rims4	-0.148	5.821	11.435	0.000721	0.031839
ENSMUSG00000036273	Lrrk2	-0.181	4.619	11.442	0.000718	0.031839
ENSMUSG00000060716	Plekhh1	-0.153	5.368	11.417	0.000728	0.032066
ENSMUSG00000022519	Srl	-0.228	3.887	11.384	0.000741	0.032367
ENSMUSG00000056553	Ptpn2	-0.099	8.228	11.388	0.000739	0.032367
ENSMUSG00000045648	Vwc2l	-0.340	3.107	11.375	0.000744	0.032386
ENSMUSG00000026496	Parp1	-0.107	7.134	11.359	0.000751	0.032574
ENSMUSG00000035621	Midn	-0.172	5.656	11.328	0.000763	0.033042
ENSMUSG00000051339	2900026A02Rik	-0.117	6.827	11.289	0.00078	0.033654
ENSMUSG00000024063	Lbh	-0.157	5.500	11.276	0.000785	0.033804
ENSMUSG00000027298	Tyro3	-0.170	7.302	11.259	0.000792	0.033872
ENSMUSG000000112441	AC122860.2	-0.954	-0.467	11.259	0.000792	0.033872

ENSMUSG00000058672	Tubb2a	-0.079	9.244	11.241	0.0008	0.03411
ENSMUSG00000053560	Ier2	-0.263	3.195	11.227	0.000806	0.034288
ENSMUSG00000000120	Ngfr	-0.509	1.209	11.219	0.00081	0.034354
ENSMUSG00000060890	Arr3	-0.528	1.127	11.185	0.000825	0.034811
ENSMUSG00000021319	Sfrp4	-0.564	0.920	11.170	0.000831	0.034974
ENSMUSG00000022415	Syng1	-0.121	8.962	11.167	0.000832	0.034974
ENSMUSG00000015745	Plekho1	-0.192	4.453	11.129	0.00085	0.035495
ENSMUSG00000021453	Gadd45g	-0.208	4.082	11.126	0.000851	0.035495
ENSMUSG00000074060	Fbxw15	-0.597	0.588	11.122	0.000853	0.035495
ENSMUSG00000031740	Mmp2	-0.386	2.113	11.109	0.000859	0.035654
ENSMUSG00000030256	Bhlhe41	-0.171	5.041	11.099	0.000864	0.035698
ENSMUSG00000035172	Plekhh3	-0.219	3.946	11.098	0.000864	0.035698
ENSMUSG00000091556	Gm14569	-0.430	1.659	11.075	0.000875	0.036064
ENSMUSG00000040659	Efh2	-0.135	7.267	10.968	0.000927	0.038016
ENSMUSG00000020432	Tcn2	-0.178	4.741	10.887	0.000969	0.039373
ENSMUSG00000021701	Plk2	-0.105	7.988	10.885	0.000969	0.039373
ENSMUSG00000027253	Lrp4	-0.149	5.423	10.873	0.000976	0.039452
ENSMUSG00000044005	Gls2	-0.188	4.481	10.874	0.000975	0.039452
ENSMUSG00000047495	Dlgap2	-0.140	5.678	10.846	0.00099	0.039793
ENSMUSG00000045659	Plekha7	-0.226	5.052	10.815	0.001007	0.040171
ENSMUSG00000021256	Vash1	-0.181	4.653	10.805	0.001012	0.040194
ENSMUSG00000024517	Grp	-0.302	4.342	10.804	0.001013	0.040194
ENSMUSG00000028680	Plk3	-0.224	3.738	10.767	0.001034	0.040919
ENSMUSG00000031642	Sh3rf1	-0.146	5.916	10.756	0.001039	0.040955
ENSMUSG00000005373	Mlxip1	-0.373	2.044	10.746	0.001045	0.040987
ENSMUSG00000023868	Pde10a	-0.139	6.239	10.749	0.001043	0.040987
ENSMUSG00000024395	Lims2	-0.243	3.541	10.720	0.00106	0.041207
ENSMUSG00000028249	Sdcbp	-0.097	8.166	10.724	0.001058	0.041207
ENSMUSG00000061751	Kalrn	-0.095	9.375	10.721	0.001059	0.041207
ENSMUSG00000063297	Luzp2	-0.154	6.971	10.729	0.001054	0.041207
ENSMUSG00000030772	Dkk3	-0.106	8.333	10.703	0.001069	0.04148
ENSMUSG00000026883	Dab2ip	-0.109	6.993	10.690	0.001077	0.041694
ENSMUSG00000041607	Mbp	-0.113	11.360	10.640	0.001106	0.042533
ENSMUSG00000020044	Timp3	-0.143	5.842	10.613	0.001123	0.04306
ENSMUSG00000029658	Wdr95	-0.599	0.594	10.609	0.001126	0.043079
ENSMUSG00000037709	Fam13a	-0.158	5.073	10.593	0.001135	0.043351
ENSMUSG00000014077	Chp1	-0.099	7.380	10.571	0.001149	0.043676
ENSMUSG00000005681	Apoa2	-1.020	-0.571	10.523	0.001179	0.044629

ENSMUSG00000025488	Cox8b	-0.898	0.507	10.509	0.001188	0.04477
ENSMUSG00000059173	Pde1a	-0.132	7.719	10.506	0.00119	0.04477
ENSMUSG00000022037	Clu	-0.090	9.807	10.499	0.001194	0.044814
ENSMUSG00000068699	Flnc	-0.377	1.945	10.475	0.00121	0.045093
ENSMUSG00000004698	Hdac9	-0.141	5.563	10.421	0.001246	0.04602
ENSMUSG00000030067	Foxp1	-0.133	6.032	10.415	0.00125	0.04602
ENSMUSG000000108616	Gm35040	-0.228	3.681	10.397	0.001262	0.046352
ENSMUSG00000021806	Nid2	-0.290	3.009	10.380	0.001274	0.046676
ENSMUSG00000096054	Syne1	-0.124	8.890	10.359	0.001288	0.047097
ENSMUSG00000090958	Lrrc32	-0.405	1.688	10.343	0.001299	0.047406
ENSMUSG00000027438	Napb	-0.104	8.889	10.320	0.001316	0.047896
ENSMUSG00000032479	Map4	-0.092	9.537	10.300	0.00133	0.048291
ENSMUSG00000039474	Wfs1	-0.190	7.269	10.295	0.001334	0.048291
ENSMUSG00000061731	Ext1	-0.163	5.360	10.294	0.001335	0.048291
ENSMUSG00000032231	Anxa2	-0.254	3.311	10.239	0.001375	0.049377
ENSMUSG00000048450	Msx1	-0.271	3.283	10.237	0.001376	0.049377
ENSMUSG000000100303	2600014E21Rik	-0.355	1.990	10.228	0.001383	0.049514
ENSMUSG00000037492	Zmat4	-0.180	5.943	10.207	0.001399	0.049868
ENSMUSG00000042156	Dzip1	-0.139	6.704	10.203	0.001402	0.049888



**Table A.5. Fear conditioned RNA-seq upregulated genes in CBP<sup>Kix/Kix</sup> mice.**

GeneID	Gene name	logFC	logCPM	LR	PValue	FDR
ENSMUSG00000069662	Marcks	0.234	7.152	53.788	2.23E-13	1.67E-10
ENSMUSG00000033717	Adra2a	0.439	4.196	53.139	3.11E-13	2.14E-10
ENSMUSG00000006930	Hap1	0.236	7.995	50.273	1.34E-12	8.21E-10
ENSMUSG000000022636	Alcam	0.222	7.089	47.588	5.26E-12	2.66E-09
ENSMUSG000000047507	Baiap3	0.423	3.925	39.779	2.84E-10	1.11E-07
ENSMUSG000000051323	Pcdh19	0.228	6.264	35.919	2.06E-09	6.80E-07
ENSMUSG000000024256	Adcyap1	0.514	2.910	34.838	3.58E-09	1.10E-06
ENSMUSG000000046447	Camk2n1	0.154	9.527	33.455	7.29E-09	2.20E-06
ENSMUSG000000026344	Lypd1	0.262	5.382	31.088	2.47E-08	6.42E-06
ENSMUSG000000021721	Htr1a	0.251	5.502	30.480	3.37E-08	8.40E-06
ENSMUSG000000025128	Bhlhe22	0.237	6.705	29.689	5.07E-08	1.25E-05
ENSMUSG000000026463	Atp2b4	0.185	7.400	29.226	6.44E-08	1.52E-05
ENSMUSG000000064373	Selenop	0.156	7.866	29.039	7.09E-08	1.65E-05
ENSMUSG000000036777	Anln	0.231	5.666	28.874	7.73E-08	1.77E-05
ENSMUSG000000032500	Dclk3	0.367	4.467	27.941	1.25E-07	2.76E-05
ENSMUSG000000039087	Rreb1	0.236	7.553	23.748	1.10E-06	0.000175
ENSMUSG000000051617	Krt9	0.343	3.702	23.511	1.24E-06	0.000194
ENSMUSG000000068735	Trp53i11	0.284	6.288	23.091	1.55E-06	0.000239
ENSMUSG000000037071	Scd1	0.158	7.719	23.033	1.59E-06	0.000244
ENSMUSG000000032690	Oas2	1.114	-0.045	22.777	1.82E-06	0.00027
ENSMUSG000000092035	Peg10	0.382	4.235	22.242	2.40E-06	0.000338
ENSMUSG000000033585	Ndn	0.306	7.400	21.293	3.94E-06	0.000498
ENSMUSG000000042793	Lgr6	0.546	1.918	21.275	3.98E-06	0.000499
ENSMUSG000000075334	Rprm	0.278	4.984	20.951	4.71E-06	0.000574
ENSMUSG000000068663	Clec16a	0.356	7.296	20.909	4.82E-06	0.000579
ENSMUSG000000028222	Calb1	0.260	7.200	20.334	6.50E-06	0.00076
ENSMUSG000000044141	E130201H02Rik	0.530	2.330	19.971	7.86E-06	0.000904
ENSMUSG000000022637	Cblb	0.191	5.518	19.361	1.08E-05	0.001192
ENSMUSG000000026043	Col3a1	0.600	1.701	19.241	1.15E-05	0.001261
ENSMUSG000000000794	Kcnn3	0.249	4.623	19.017	1.30E-05	0.001358
ENSMUSG000000021803	Cdhr1	0.311	3.598	18.972	1.33E-05	0.001381
ENSMUSG000000055670	Zzef1	0.192	6.238	18.748	1.49E-05	0.001517
ENSMUSG000000021032	Ngb	0.489	2.105	18.714	1.52E-05	0.001535
ENSMUSG000000004902	Slc25a18	0.168	5.909	18.215	1.97E-05	0.001925
ENSMUSG000000027861	Casq2	0.669	1.073	18.004	2.20E-05	0.002081

ENSMUSG00000047181	Samd14	0.169	5.930	17.886	2.35E-05	0.002191
ENSMUSG00000047238	Mageh1	0.195	5.391	17.630	2.68E-05	0.002427
ENSMUSG00000042607	Asb4	1.070	-0.154	17.609	2.71E-05	0.002441
ENSMUSG00000028360	Slc44a5	0.177	5.642	17.434	2.97E-05	0.002608
ENSMUSG00000087075	A230065H16Rik	0.605	1.532	17.185	3.39E-05	0.00287
ENSMUSG00000023886	Smoc2	0.179	5.531	17.125	3.50E-05	0.002948
ENSMUSG00000020848	Doc2b	0.240	7.127	16.825	4.10E-05	0.003339
ENSMUSG00000045573	Penk	0.227	5.001	16.722	4.33E-05	0.003459
ENSMUSG00000021136	Smoc1	0.274	3.854	16.563	4.71E-05	0.003709
ENSMUSG00000070695	Cntnap5a	0.213	5.193	16.547	4.75E-05	0.003724
ENSMUSG00000020846	Rflnb	0.309	4.148	16.508	4.85E-05	0.003767
ENSMUSG00000090946	Ccdc71l	0.187	5.221	16.418	5.08E-05	0.003904
ENSMUSG00000030592	Ryr1	0.195	5.716	16.268	5.50E-05	0.00418
ENSMUSG00000063626	Unc5d	0.204	4.965	15.957	6.48E-05	0.004819
ENSMUSG00000005892	Trh	1.077	0.750	15.909	6.65E-05	0.004923
ENSMUSG00000079845	Xlr4a	0.858	0.550	15.391	8.74E-05	0.00623
ENSMUSG00000069049	Eif2s3y	0.164	5.778	15.213	9.61E-05	0.006724
ENSMUSG00000020123	Avpr1a	0.942	-0.039	15.060	0.000104	0.007157
ENSMUSG00000022483	Col2a1	0.454	2.041	15.039	0.000105	0.00721
ENSMUSG00000043498	9330132A10Rik	0.189	6.141	14.932	0.000111	0.007454
ENSMUSG00000022311	Csmd3	0.202	4.774	14.887	0.000114	0.007562
ENSMUSG00000029563	Foxp2	0.649	0.959	14.856	0.000116	0.007641
ENSMUSG00000020641	Rsad2	0.664	0.789	14.826	0.000118	0.007731
ENSMUSG00000041378	Cldn5	0.205	5.388	14.762	0.000122	0.007939
ENSMUSG00000067594	Krt77	0.261	3.674	14.646	0.00013	0.00835
ENSMUSG00000052684	Jun	0.161	7.076	14.580	0.000134	0.008551
ENSMUSG00000084979	Gm16267	0.609	0.903	13.889	0.000194	0.0119
ENSMUSG00000028047	Thbs3	0.201	5.500	13.793	0.000204	0.01239
ENSMUSG00000020061	Mybpc1	0.598	1.046	13.775	0.000206	0.012465
ENSMUSG00000036198	Arhgap36	0.688	0.666	13.657	0.000219	0.013144
ENSMUSG00000038173	Enpp6	0.277	3.400	13.531	0.000235	0.013763
ENSMUSG00000032268	Tmprss5	0.520	1.456	13.523	0.000236	0.013771
ENSMUSG00000040929	Rfx3	0.189	6.602	13.457	0.000244	0.014173
ENSMUSG00000086290	Snhg12	0.247	3.780	13.424	0.000248	0.01423
ENSMUSG00000090863	A530084C06Rik	0.869	0.072	13.323	0.000262	0.014864
ENSMUSG00000028019	Pdgfc	0.314	2.948	13.231	0.000275	0.015561
ENSMUSG00000036902	Neto2	0.157	5.653	13.100	0.000295	0.016319
ENSMUSG00000058626	Capn11	1.307	2.168	13.070	0.0003	0.016522

ENSMUSG00000010663	Fads1	0.106	8.093	12.959	0.000318	0.017311
ENSMUSG00000044244	Il20rb	0.421	2.001	12.805	0.000346	0.018622
ENSMUSG00000070003	Ssbp4	0.128	7.038	12.667	0.000372	0.019864
ENSMUSG00000036357	Gpr101	0.281	3.689	12.607	0.000384	0.020384
ENSMUSG00000043872	Zmym1	0.227	4.437	12.608	0.000384	0.020384
ENSMUSG00000041798	Gck	0.438	1.889	12.591	0.000388	0.020488
ENSMUSG00000023048	Prr13	0.172	5.231	12.542	0.000398	0.02091
ENSMUSG00000023191	P3h3	0.182	5.216	12.472	0.000413	0.021572
ENSMUSG00000050953	Gja1	0.113	7.907	12.439	0.000421	0.021765
ENSMUSG00000041459	Tardbp	0.101	7.895	12.333	0.000445	0.022713
ENSMUSG00000036782	Klhl13	0.171	5.115	12.325	0.000447	0.022726
ENSMUSG00000024548	Setbp1	0.154	5.411	12.234	0.000469	0.02364
ENSMUSG00000042353	Frem3	0.604	0.803	12.132	0.000496	0.024684
ENSMUSG00000034918	Cdhr2	0.726	0.194	12.123	0.000498	0.024727
ENSMUSG00000052155	Acvr2a	0.136	6.072	12.117	0.0005	0.024745
ENSMUSG00000050122	Vwa3b	0.221	4.326	12.050	0.000518	0.025206
ENSMUSG00000059674	Cdh24	0.242	4.548	12.029	0.000524	0.025423
ENSMUSG00000013584	Aldh1a2	0.396	2.238	11.867	0.000571	0.027378
ENSMUSG00000049583	Grm5	0.101	8.069	11.841	0.000579	0.027626
ENSMUSG00000045827	Serpinb9	0.297	2.940	11.801	0.000592	0.027949
ENSMUSG00000033849	B3galt2	0.206	5.598	11.712	0.000621	0.028582
ENSMUSG00000030029	Lrig1	0.169	5.120	11.636	0.000647	0.029401
ENSMUSG00000022510	Trp63	0.468	1.471	11.503	0.000695	0.031182
ENSMUSG00000031075	Ano1	0.291	2.951	11.503	0.000695	0.031182
ENSMUSG00000032024	Clmp	0.188	4.910	11.509	0.000693	0.031182
ENSMUSG00000013846	St3gal1	0.325	5.410	11.401	0.000734	0.032256
ENSMUSG00000024008	Cpne5	0.183	4.819	11.381	0.000742	0.032367
ENSMUSG00000036356	Csgalnact1	0.240	4.153	11.269	0.000788	0.033848
ENSMUSG00000027078	Ube2l6	0.405	1.894	11.212	0.000813	0.034396
ENSMUSG00000098557	Kctd12	0.139	6.382	11.132	0.000849	0.035495
ENSMUSG00000031671	Setd6	0.220	3.976	10.972	0.000925	0.038016
ENSMUSG00000024529	Lox	0.902	-0.444	10.912	0.000955	0.039082
ENSMUSG00000028749	Pla2g2f	0.374	2.055	10.903	0.00096	0.03918
ENSMUSG00000034098	Fstl5	0.166	4.933	10.861	0.000982	0.039614
ENSMUSG00000039997	Ifi203	0.319	2.486	10.844	0.000991	0.039793
ENSMUSG00000023795	Pisd-ps2	0.192	6.868	10.837	0.000995	0.039855
ENSMUSG00000020810	Cygb	0.145	5.735	10.814	0.001008	0.040171
ENSMUSG00000024524	Gnal	0.107	7.072	10.758	0.001038	0.040955

ENSMUSG00000039910	Cited2	0.183	4.912	10.677	0.001085	0.041893
ENSMUSG00000034163	Zfc3h1	0.120	6.321	10.665	0.001092	0.042075
ENSMUSG00000032878	Ccdc85a	0.120	6.840	10.580	0.001143	0.043559
ENSMUSG00000037681	Esyt3	0.308	2.568	10.532	0.001173	0.044494
ENSMUSG00000042063	Zfp386	0.234	3.519	10.505	0.001191	0.04477
ENSMUSG00000052906	Ubxn8	0.167	4.843	10.488	0.001202	0.044982
ENSMUSG00000015085	Entpd2	0.272	3.094	10.476	0.00121	0.045093
ENSMUSG00000027637	1110008F13Rik	0.217	4.091	10.436	0.001236	0.045958
ENSMUSG00000027313	Chac1	0.306	2.586	10.431	0.001239	0.045996
ENSMUSG00000025498	Irf7	0.503	1.064	10.414	0.001251	0.04602
ENSMUSG00000028037	Ifi44	0.962	-0.795	10.421	0.001246	0.04602
ENSMUSG00000104459	Gm37824	0.363	1.942	10.287	0.00134	0.048373
ENSMUSG00000060550	H2-Q7	0.670	0.314	10.245	0.00137	0.049365
ENSMUSG00000026312	Cdh7	0.295	3.003	10.219	0.00139	0.049662

**Figure A.1**

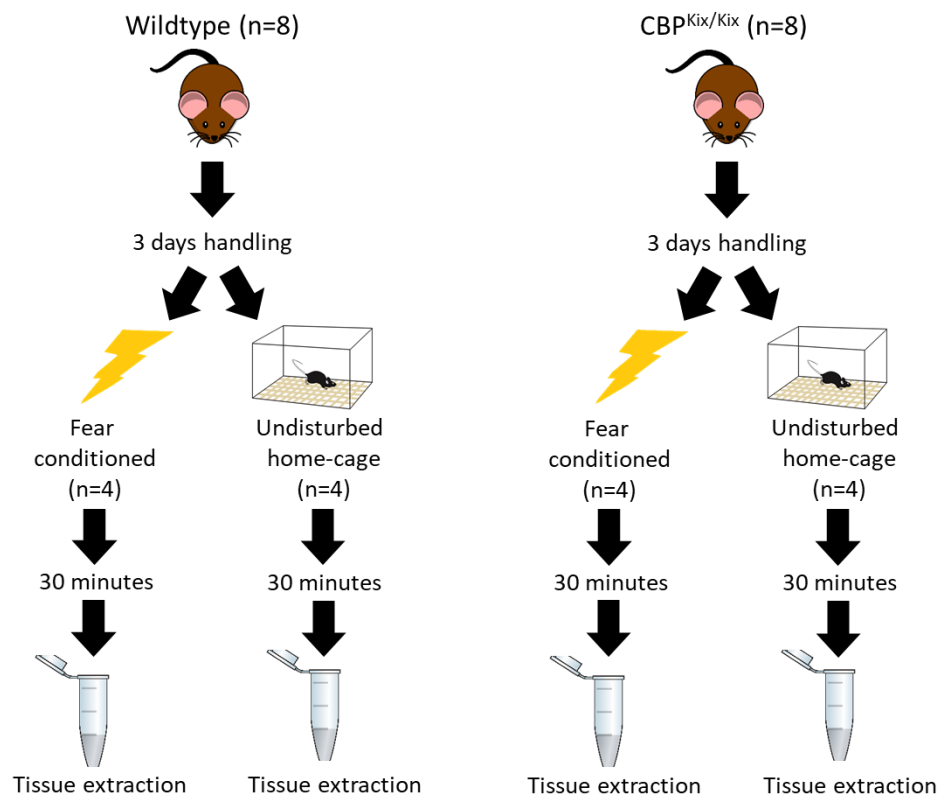


Figure A.2

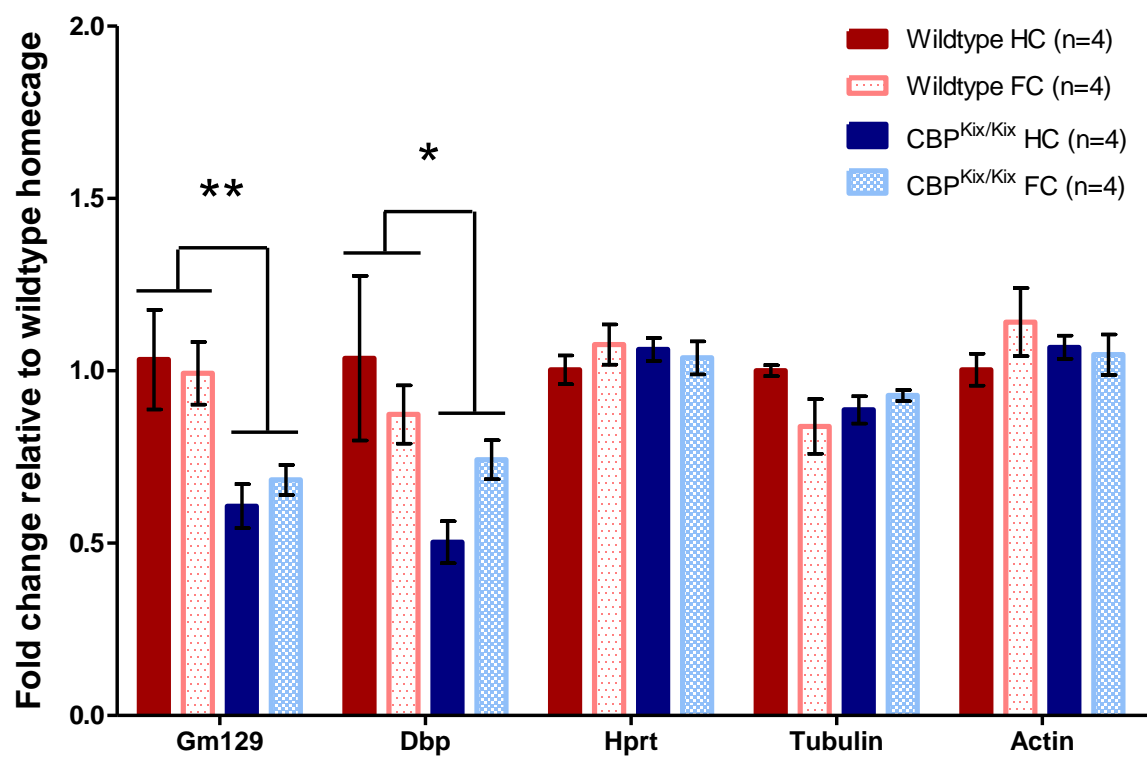


Figure A.3

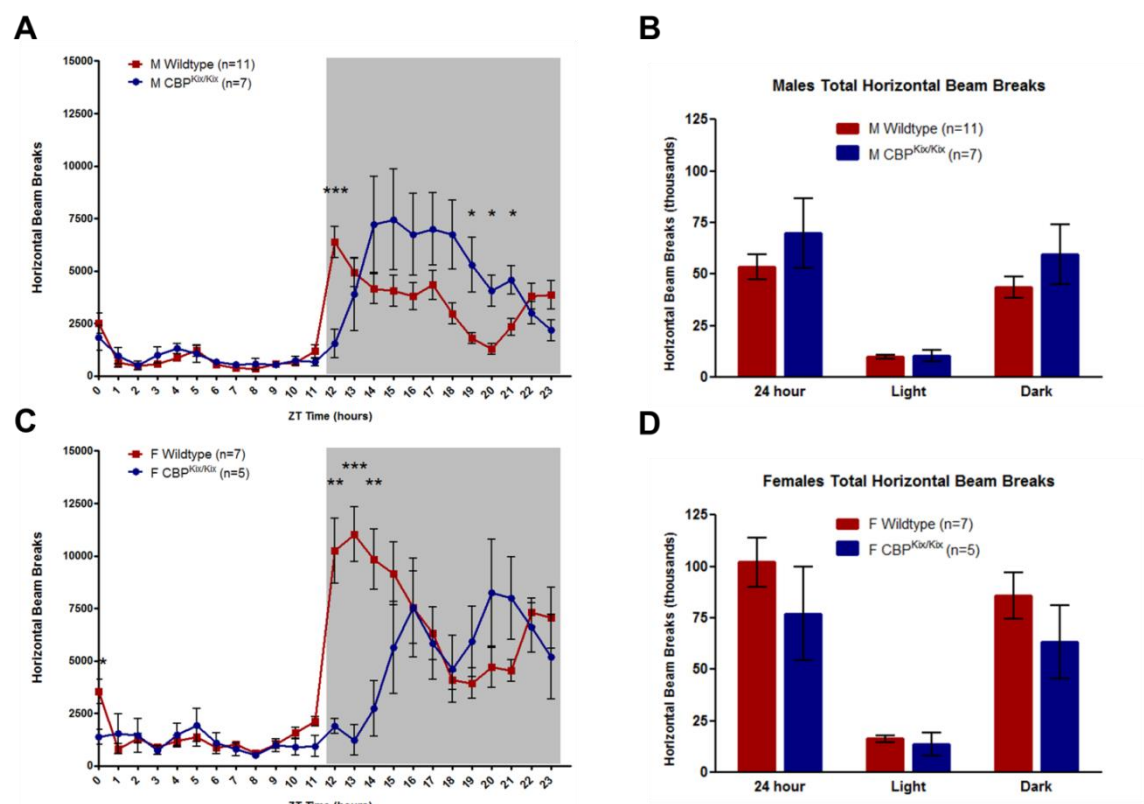
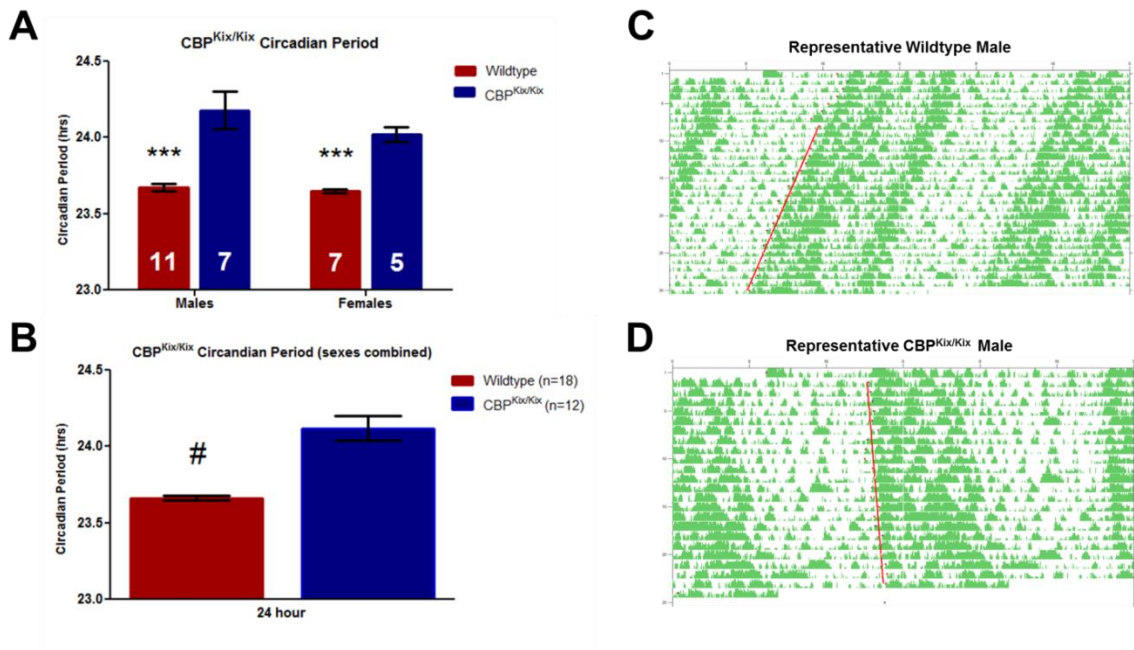
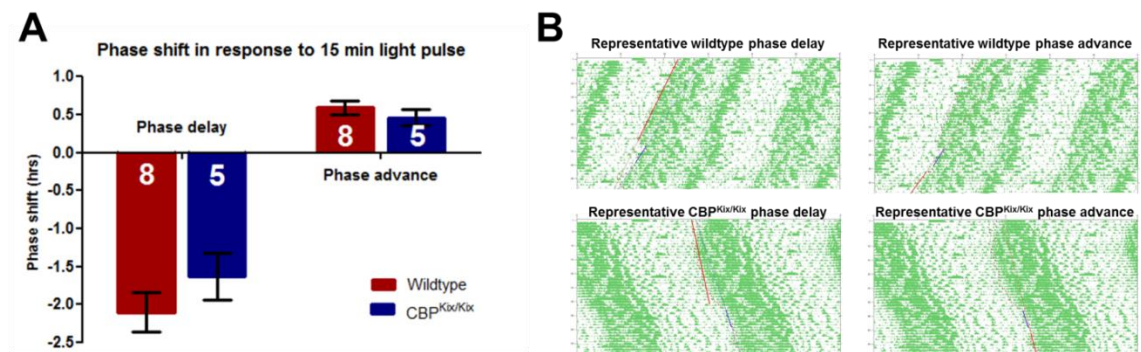


Figure A.4





**Figure A.5**



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